



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Roberts
Serial No. 09/775,909
Filed: February 2, 2001
For: VACCINE
COMPOSITIONS

DECLARATION

I, Mark Roberts, declare as follows:

1. I am the inventor of this patent application. I currently hold the position of Professor of Molecular Bacteriology, Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK. My Research interests are focused on understanding, at the molecular level, the mechanisms by which pathogens cause disease and the nature of the immune response to them. A copy of my curriculum vitae is attached as Exhibit 1.
2. I have been asked to comment on the Examiner's rejection of certain claims as being unpatentable (obvious) over Wilson et al in view of Nencioni et al, as set forth on pages 5 and 6 of the Official Action of 19 April 2004. I confirm that I have read the Official Action, the patent application, and Wilson et al and Nencioni et al.
3. My main comment is that it cannot be concluded from Wilson et al that the adjuvant activity of pertussis toxin is independent of the enzymatic activity of the toxin. There are two reasons for this.
4. The first reason derives from the fact that pertussis toxin produces a myriad of biological effects by catalysing the ADP-ribosylation of many different G proteins (Ui, 1988, The multiple biological activities of pertussis toxin, In Pathogenesis and immunity in pertussis, edited by A.C. Wardlaw and R. Parton, pages 121-145, Exhibit 2). Wilson et al tried to examine just one of these effects, namely the elevation of cAMP levels. Thus, even if Wilson et al did show that elevation of cAMP has no effect on adjuvant activity (which they did not for reasons which I explain below), this would not allow any conclusion to be drawn that the adjuvant activity of pertussis toxin is independent of its enzymatic activity. The most that could be concluded is that the adjuvant activity of pertussis toxin is probably mediated through an effect of its enzymatic activity different from its effect on cAMP levels. This is recognised in the last paragraph of the Discussion section of Wilson et al, where it is stated that:

Although this experiment is a rather blunt probe of immune regulation we consider that CT and PT may act by an alternative mechanism, such as via a common G protein-mediated effect not involving enhancement of adenylate cyclase activity.

5. Thus, even the authors of Wilson et al recognise that, even if their results are taken at face value, the effect of pertussis toxin on immune regulation is likely to derive from a G-protein mediated effect (i.e. an enzyme-mediated effect) of the toxin not involving elevation of cAMP. In other words, the authors recognise that the effect of pertussis toxin on immune regulation is likely to derive from one of the myriad of non-cAMP related effects resulting from the enzymatic activity of pertussis toxin.
6. The second reason why it cannot be concluded from Wilson et al that the adjuvant activity of pertussis toxin is independent of the enzymatic activity of the toxin is that Wilson did not in fact show that they had any produced any effect on cAMP levels. The relevant experiment described in Wilson et al involves feeding forskolin to mice. Forskolin is known to raise cAMP levels in cultured cells *in vitro*. However, Wilson et al does not show that feeding forskolin to mice produces elevated cAMP levels or has any other relevant effect. All that Wilson et al showed was that the mice got sick. They did not show that feeding forskolin had any relevant effect on the immune function of the mice.
7. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of this Declaration, the patent application, or any patents issuing thereon.

Declared this day of September 2004

Mark Roberts

CURRICULUM VITAE

Name: Mark ROBERTS

Date of Birth: 25th July, 1959

Nationality: British

PRESENT POSITION From 12/1994

Professor of Molecular Bacteriology, Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow University of Glasgow Veterinary School, Bearsden Rd, Glasgow G61 1QH

PREVIOUS EMPLOYMENT:

1976-1981 Medical Laboratory Scientific Officer, Department of Microbiology, St. Ann's General Hospital

1988-1991 Bacteriologist, Department of Molecular Biology, Wellcome Biotech, Langley Court, Beckenham, Kent.

1991-1994 Research Manager, Vaccine Research Unit, Medeva Group Research, Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY.

EDUCATION:

1970-1976 Creighton Comprehensive School

1976-1978 Tottenham Technical College
ONC Medical Laboratory Sciences

1978-1980 Harrow College of Higher Education
HNC Medical Laboratory Subjects

1981-1984 University of East Anglia
BSc. Biological Sciences (1st Class Hons.)

1984-1988 University of Leicester
Ph.D. CASE studentship with Unilever, Colworth House

Ph.D. Project: "Role of the aerobactin-mediated iron uptake system in the pathogenesis of *Escherichia coli* infections"

Editorial Board Infection and Immunity

PUBLICATION LIST

1. Williams, P.H. and Roberts, M. 1985. Aerobactin-mediated iron uptake: a virulence determinant in enteropathogenic *Escherichia coli*? *Lancet* i: 736.
2. Roberts, M., Leavitt, R.W., Carbonetti, N.H., Ford, S., Cooper, R.A. and Williams, P.H. 1986. RNA-DNA hybridization analysis of transcription of the plasmid ColV-K30 aerobactin gene cluster. *J.Bacteriol.* 167: 467-472.
3. Roberts, M., ParthaSarathy, S., Lam-Po-Tan, M.K.L. and Williams, P.H. 1986. The aerobactin iron uptake system in enteropathogen *Escherichia coli*: evidence for an extinct transposon. *FEMS Micro.Letts.* 37: 215-219.
4. Linggood, M.A., Roberts, M., Ford, S., Parry, S.H. and Williams, P.H. 1987. Incidence of the aerobactin iron uptake system among *Escherichia coli* isolates from infections of farm animals. *J.Gen.Microbiol.* 135: 835-842.
5. Roberts, M., Roberts, I., Korhonen, T.K., Jann, K., Bitter-Suermann, D., Boulnois, G.J. and Williams, P.H. 1988. DNA probes for K-antigen (Capsule) typing of *Escherichia coli*. *J.Clin.Microbiol.* 26: 385-387.
6. Williams, P.H., Roberts, M. and Hinson, G. 1988. Stages in bacterial invasion. *J.Appl.Bact.Symp.Suppl.* 131S-147S.
7. Mark Roberts. Aerobactin in human and animal disease. 1988. PhD. Thesis. University of Leicester.
8. Williams, P.H. and Roberts, M. 1989. Iron scavenging in the pathogenesis of *Escherichia coli*. P331-350. In "Genetics of Bacterial Diversity". Eds. D.A. Hopwood and K.F. Chater. Academic Press, London.
9. Roberts, M., Woodridge, K.G., Gavine, H., Kuswandi, S.I. and Williams, P.H. 1989. Inhibition of biological activities of the aerobactin receptor protein in rough strains of *Escherichia coli* by polyclonal antiserum raised against native protein. *J.Gen.Microbiol.* 135: 2387-2398.
10. Derbyshire, P., Baldwin, T., Stevenson, P., Griffiths, E., Roberts, M., Williams, P., Hale, T.L. and Formal, S.B. 1989. Expression in *Escherichia coli* K-12 of the 76,000 dalton iron-regulated outer membrane protein of *Shigella flexneri* confers sensitivity to cloacin DF13 in the absence of *Shigella* O antigen. *Infect.Immun.* 57: 2794-2798.
11. Roberts, M., Maskell, D., Novotny, P. and Dougan, G. 1990. Construction and characterization *in vivo* of *Bordetella pertussis aroA* mutants. *Infect.Immun.* 58: 732-739.
12. Dougan, G., Chatfield, S.N., Roberts, M., Charles, I., Comerford, S., Jing Li, L. and Fairweather, N.F. 1990. Bacterial pathogens - A route to oral drug delivery. *Biochem.Soc.Trans.* 18: 746-748.

13. Strugnelli, R., Roberts, M. and Dougan, G. 1990. Rationally attenuated pathogens as vaccines and antigen carriers. *Life Science* 2: 30-36.
14. Bhargava, A., Leininger, E., Roberts, M., Li, Z.M., Stibitz, S., Charles, I., Fairweather, N., Novotny, P., Manclark, C. and Brennan, M. 1990. Filamentous hemagglutinin and the 69-kDa protein, pertactin, promote adherence of *Bordetella pertussis* to epithelial cells and macrophages. pp.137. Sixth Int. Symp. Pertussis.
15. Roberts, M., De Azavedo, J., Leininger, E., Brennan, M.J., Hewlett, E.L., Robinson, A., Fairweather, N.F., Dougan, G., Novotny and Charles, I.G. 1990. Construction and characterisation of *Bordetella pertussis* 69-kDa mutants. pp.139. Sixth Int. Symp. Pertussis.
16. O'Gaora, P., Roberts, M., Bowe, F., Hommaeche, C., DeMarco de Hommaeche, R., Cafferkey, M., Tite, J. and Dougan, T. 1990. *Yersinia enterocolitica* aroA mutants as carriers of the B subunit of the *Escherichia coli* heat labile enterotoxin to the murine immune system. *Microb.Path.* 9: 105-116.
17. Roberts, M. and Dougan, G. 1990. Rationally attenuated bacteria as live vaccines and carriers of foreign antigen determinants. *J.Biomed.Sci.*
18. Streulins, M., Mondal, G., Roberts, M. and Williams, P.H. 1990. Role of bacterial and host factors in the pathogenesis of *Shigella* septicemia. *Eur.J.Clin.Microbiol.Infect.Dis.* 9: 337-344.
19. Fairweather, N., Chatfield, S.N., Charles, I.G., Roberts, M., Lipscombe, M., Jing Li, L., Strugnelli, R., Comerford, S., Tite, J. and Dougan, G. 1990. Use of live, attenuated bacteria to stimulate immunity. *Research in Microbiology* 141: 769-774.
20. Chatfield, S.N., Fairweather, N., Roberts, M., Charles, I., Tite, J. and Dougan, G. 1990. Rationally attenuated *Salmonella* strains as live oral vaccines. p.182-185. In: "5th European Congress on Biotechnology". Ed. C. Christiansen, L. Munck & J. Villad. Publish. Munksgaard International, Copenhagen
21. Fairweather, N.F., Makoff, A.J., Charles, I.G., Oxe, M.D., Ballantine, S., Roberts, M. and Dougan, G. 1990. *Bordetella pertussis* P.69: High-level expression, purification and characterisation of recombinant P.69 in *Escherichia coli*. p.275-280. Proceedings of the Sixth Int. Symp. on Pertussis.
22. Leininger, E., Roberts, M., Kenimer, J.G., Charles, I.G., Fairweather, N., Novotny, P. and Brennan, M.J. 1991. Pertactin (P.69) an arginine-glycine-aspartic acid containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc.Natl.Acad.Sci. USA* 88: 345-349.
23. Chatfield, S.N., Fairweather, N., Tite, J., Charles, I., Roberts, M., Posada, M., Strugnelli, R. and Dougan, G. 1991. The development of genetically defined live bacterial vaccines. p.279-285. In: "Molecular Pathogenesis of Gastrointestinal Infections". Ed. T. Wadstrom *et al.*

24. Dougan, G., Roberts, M. and Chatfield, S.N. 1991. The influence of genetics on the development of oral vaccines. *Biotech.* 6: 49-52.
25. Roberts, M., Fairweather, N.F., Leininger, E., Pickard, D., Hewlett, E.L., Robinson, A., Hayward, C., Dougan, G. and Charles, I.G. 1991. Construction and characterisation of *Bordetella pertussis* mutants lacking the vir-regulated P.69 outer membrane protein. *Mol.Microbiol.* 5: 1391-1404.
26. Lipscombe, M., Charles, I.G., Roberts, M., Dougan, G., Tite, J. and Fairweather, N.F. 1991. Intranasal immunization using the B subunit of the *Escherichia coli* heat labile toxin fused to an epitope of the *Bordetella pertussis* P.69 antigen. *Mol.Microbiol.* 5: 1385-1392.
27. Charles, I.G., JingLi, L., Roberts, M., Beasley, K., Romanos, M., Pickard, D.J., Francis, M., Campbell, D., Dougan, G., Brennan, M.J., Manclark, C.R., Au Jensen, M., Heron, I., Chubb, A., Novotny, P. and Fairweather, N.F. 1991. Identification and characterisation of a protective immunodominant B cell epitope of pertactin (P.69) from *Bordetella pertussis*. *Eur.J.Immunol.* 21: 1147-1153.
28. Roberts, M., Maskell, D., Novotny, P. and Dougan, G. 1991. Construction and characterisation *in vivo* of *B.pertussis* *aroA* mutant strains. *Proceedings of European Workshop on Pertussis.* Eds. Corbel, M.J. and Redhead, K.J. *Med.Microbiol.* 35: 186-187.
29. Roberts, M., Tite, J.P., Fairweather, N.F., Dougan, G. and Charles, I.G. 1992. Recombinant P.69/pertactin: Immunogenicity and protection of mice against *B.pertussis* infection. *Vaccine* 10: 43-48.
30. Strugnell, R., Dougan, G., Chatfield, S., Charles, I., Fairweather, N., Tite, J., Li, J-L, Beesley, J. and Roberts, M. 1992. Characterisation of a *Salmonella typhimurium* *aro* vaccine strain expressing P.69 antigen of *Bordetella pertussis*. *Infect. Immun.* 60: 3994-4002.
31. Chatfield, S., Roberts, M., Londono, P., Douce, G. and Dougan, G. 1993. The Development of oral vaccines based on live attenuated *Salmonella* strains. *FEMS Immunol and Med. Microbiol.* 7: 1-8.
32. Roberts, M., Cropley, I., Chatfield, S. and Dougan, G. 1993. Protection of mice against respiratory *Bordetella pertussis* infection by intranasal immunisation with P.69 and FHA. *Vaccine*, 11: 866-872.
33. Dougan, G., Roberts, M., Douce, G., Londono, P., Hormaeche, C.E., Harrison, J. and Chatfield, S. 1993. The genetics of *Salmonella* and vaccine development p323-332. In: *The Biology of Salmonella.* Eds. Cabello, F., Hormaeche, C., Mastroeni, P. and Bonina, L. Plenum Press, New York and London.
34. Roberts, M., Dougan, G., Li, J-L., Chatfield, S. and Strugnell, R. 1993. Immunisation against *Bordetella pertussis* infection of mice using a *Salmonella*

- typhimurium aro* vaccine strain expressing the *Bordetella pertussis* P.69 antigen. Recent progress in pertussis research. *Biologicals* 21: 4 - 5.
35. Roberts, M., Cropley, I., Chatfield, S., Douce, G. and Dougan, G. 1993. Protection of mice against *Bordetella pertussis* infection by intranasal immunisation with P.69 or FHA. Recent progress in pertussis research. *Biologicals* 21: 40.
 36. Roberts, M., Chatfield, S.N. and Dougan, G. 1994. *Salmonella* as carriers of heterologous antigens. p27-58. In: Novel delivery systems for oral vaccines. Ed. D. T. O'Hagan. CRC Press, Boca Raton, FL.
 37. Charles, I.G., Rodgers, B.C., Musgrave, S., Peakman, T.C., Makoff, A.J., Chubb, A.J., Dougan, G. and Roberts, M. 1993. Expression of P.69 pertactin from *Bordetella pertussis* in a baculovirus/insect cell system: protective properties of recombinant protein. *Research in Microbiology* 144: 691-690.
 38. Douce, G., Cropley, I., Roberts, M., Fairweather, N., Chatfield, S. and Dougan, G. 1994. A role for toxins in the development of oral vaccines. P474. *Bacterial Protein Toxins. Zbl. Bakt. Suppl.* 24. Eds Feer et al. Gustav Fischer, Stuttgart, 1994.
 39. Charles, I., Fairweather, N., Pickard, D., Beesley, J., Slater, D., Dougan, G. and Roberts, M. 1994. Expression of the *Bordetella pertussis* pertactin adhesin in *Escherichia coli*: Fate of the carboxy-terminal domain. *Microbiology* 140: 3301-3308.
 40. Chatfield, S.N., Dougan, G. and Roberts, M. 1994. Progress in the development of multivalent oral vaccines based on live attenuated *Salmonella*. p55-86. In: *Recombinant vaccines: Modern vaccinology*. Ed. Kurstak, E.. Plenum Medical, New York
 41. Chatfield, S.N., Roberts, M., Jingli Li., Starns, A. and Dougan, G. 1994. The use of live attenuated salmonella for oral vaccination. p35-42. In: *Recombinant In: Vaccines in Vaccine development*. Ed., Brown, F. Dev Biol. Stand. 82.
 42. Pickard, D., Li, J., Roberts, M., Maskell, D., Hone, D., Levine, M., Dougan, G. and Chatfield, S. 1994. Characterisation of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. *Infect. Immun.* 62: 3984-3993.
 43. Douce, G., Turcotte, C., Roberts, M., Pizza, M., Domenghini, M., Rappuoli, R. and Dougan G. 1995. Mutants of *E. coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as non-toxic mucosal adjuvants. *P.N.A.S* 92: 1644-1648.
 44. Chatfield, S.N., Roberts, M., Dougan, G., Hormaeche, C. and Khan, C.M.A. 1995. The development of oral vaccines against parasitic diseases utilizing live attenuated *Salmonella*. *Parasitol.* 110: S17-S24.
 45. Roberts, M., Bacon, A., Rappuoli, R., Pizza, M., Cropley, I., Douce, G., Dougan, G., Mariarosaria, M., McGhee and Chatfield, S. 1995. A mutant pertussis toxin molecule

- that lacks ADP-ribosyltransferase activity, PT-9K/129G, is an effective mucosal adjuvant for intranasally delivered proteins. *Infect. Immun.* 63: 2100-2108.
46. Cropley, I., Douce, G., Roberts, M., Chatfield, S., Rappuoli, R., Pizza, M., and Dougan, G. 1995. Mucosal and systemic immunogenicity of a recombinant, non ADP-ribosylating pertussis toxin: Comparison of native and formaldehyde-treated proteins. *Vaccine* 13: 1731-1816.
 47. Hormaeche, C. E., Anjam Khan, C. M., Mastroenii, P., Villareal, B., Roberts, M., Dougan, G., and Chatfield, S. 1995. *Salmonella* vaccines: mechanisms of immunity and their use as carriers of recombinant antigens. pp119 - 154 in "Molecular and Clinical Aspects of Bacterial Vaccine Development". Eds D. A. Ala' Aldeen and C. E. Hormaeche. John Wiley & Sons Ltd., Chichester.
 48. Kingsley, R., Rabsch, W., Stephens, P., Roberts, M., Reissbrodt, R. and Williams, P.H. 1995. Iron supplying systems of *Salmonella* in diagnostics, epidemiology and infection. *FEMS Immunol. and Med. Microbiol.* 11: 257 - 264.
 49. VanCott, J.L., Okahashi, N., Yamamoto, T., Marinaro, M., Roberts, M., Chatfield, S.N., Pascual, D.W., Kiyono, H. and McGhee, J. 1995. Th2 phenotype is expressed by high oral dose and Th1 phenotype by is expressed by low oral dose of recombinant *Salmonella* in IFN- $\gamma^{-/-}$ gene disrupted mice. *Clin. Immunol. Immunopathol* 76:1 S6.
 50. VanCott, J.L., Marinaro, M., Roberts, M., Chatfield, S.N., T., Yamamoto, Hiroi K., Fujihashi, H., Kiyono, H. and McGhee, J. 1995. Importance of vaccine delivery mode on outcome of helper T subsets and cytokines for mucosal IgA responses. In: *Recent Advances in Otitis Media*,
 51. VanCott, J.L., Staats, H.F., Pascual, D.W., Roberts, M., Chatfield, S.N., Yamamoto, M., Carter, P.B., Kiyono, H. and McGhee, J. 1995. Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages and derived cytokines following oral immunisation with live recombinant *Salmonella*. *J. Immunol.* 156: 1504-1514
 52. VanCott, J.L., Coste, M., Chatfield, S., Roberts, M., Pascual, D.W., Kiyono, H. and McGhee, J. 1995. Loss of resistance of to attenuated recombinant *Salmonella* in IFN- $\gamma^{-/-}$ disrupted mice. *FASEB J.* 9: A810.
 53. Okahashi, N., Yamamoto, T., VanCott, J.L., Chatfield, S.N., Roberts, M., Bluthmann, H., Hiroi, T., Kiyono, H. and McGhee, J. 1995. Oral immunisation of IL-4 knockout mice with *Salmonella* or cholera toxin reveals that CD4+ Th2 cells producing IL-6 and IL-10 are associated with mucosal IgA responses. *Infect. Immun.* 64: 1516-1525.
 54. Anderson, R., Dougan, G and Roberts, M. 1996. Delivery of the Pertactin/P.69 polypeptide of *Bordetella pertussis* using an attenuated *Salmonella typhimurium* vaccine strain: expression levels and immune response. *Vaccine* 14:1384-1390.
 55. Everest, P., Jingli, L., Douce, G., Charles, I., De Azavedo, J., Chatfield, S., Dougan, G. and Roberts, M. 1996. Role of the *Bordetella pertussis* .69/Pertactin protein and the

- P.69/Pertactin RGD motif in the Adherence to and Invasion of Mammalian cells. Microbiol. 142: 3261-3268.
56. Anderson, R., Xiao-Ming, G., Papakonsantinopoulou, A., Roberts, M and Dougan, G. 1996. Immune response in mice following immunisation with DNA encoding fragment C of tetanus toxin. Infect. Immun. 64: 3168-3173.
57. Kingsley, R., Rabsch, W., Roberts, M., Reissbrodt, R. and Williams, P.H. 1996. TonB-dependent iron supply in *Salmonella* by α -ketoacids and α -hydroxyacids. FEMS Microbiol. Letts. 140: 65 - 70.
58. Douce, G., Dougan, G., Pizza, M.G., Rappuoli R. and Roberts, M. 1997. Mutant pertussis and *Escherichia coli* heat-labile toxins as adjuvants for enhancing local and systemic immune responses to co-administered non-living antigens. pp 253 – 262. In: "New Generation Vaccines" Eds. M.M. Levine, Woodrow, G.C., Kaper, J.B. and Cobon, G.S. Marcel Dekker Inc. New York.
59. Reissbrodt, R., Kingsley, R., Rabasch, W., Beer W., Roberts, M and Williams, P. H. 1997. Iron regulated excretion of α -keto acids by *Salmonella typhimurium*. J.Bacteriol. 179: 4538 - 4544.
60. Everest, P., Allen, J., Papakonsantinopoulou, A., Mastroeni, P., Roberts, M and Dougan, G. 1997. *Salmonella typhimurium* infections in mice deficient in interleukin 4 production. J. Immunol. 159: 1820-1827.
61. Everest, P., Roberts, M. and Dougan, G. 1998. Susceptibility of mice deficient in Tumour Necrosis Factor α receptor to *Salmonella typhimurium*. Infect. Immun. 66: 3355-3364.
62. Roberts, M., Li, J., Bacon, A. and Chatfield, S. 1998. Oral vaccination against tetanus: comparison of the immunogenicity of *Salmonella* strains expressing fragment C from the *nirB* and *htrA* promoters. Infect. Immun. 66: 3080-3087.
63. Roberts, M. 1998. Host Reaction – Animals. Introduction. p487 –489 In "Bacterial Pathogenesis, Methods in Microbiology, Vol. 27". Eds. Williams, P, Ketley, J. and Salmond, G. Academic Press Ltd.
64. Everest, P., Roberts, M. and Dougan, G. 1998. Strategies for the use of gene knockout mice in the investigation of bacterial pathogenesis and immunology. p527-535. In "Bacterial Pathogenesis, Methods in Microbiology, Vol. 27". Eds. Williams, P, Ketley, J. and Salmond, G. Academic Press Ltd.
65. VanCott, J.L., Chatfield, S.N., Roberts, M., Hone, D., Hohmann, E., Pascual, D.W., Yamamoto, M., Yamamoto, S. and McGhee, J. R. 1998. Regulation of attenuation and toxicity to bacteria by modulation of virulence genes. Nature Medicine 11: 1247 – 1252.

66. Humphreys, S., Stevenson, A., Bacon, A., Weinhardt, A.B. and Roberts, M. 1999. The alternative sigma factor, σ^E , is critically important for the virulence of *Salmonella typhimurium*. Infect. Immun. 67:1560-1568.
67. Kingsley, R.A., Reissbrodt, R., Rabsch, W., Ketley, J.M., Tsolis, R.M., Everest, P., Dougan, G., Baumler, A.J., Roberts, M. and Williams P.H. 1999. Ferrioxamine-mediated Iron(III) utilization by *Salmonella enterica*. Appl. Environ. Microbiol. 65: 1610-18.
68. Roberts, M., Bacon, A., Li, J. and Chatfield, S. 1999. Prior immunity to homologous and heterologous *Salmonella* serotypes suppresses the local and systemic anti-fragment C antibody response and protection from tetanus toxin in mice immunised with *Salmonella* strains expressing fragment C. Infect. Immun. 67:3810-3815.
69. Forde, C. B., Shi, X. Li J and Roberts, M. 1999. *Bordetella bronchiseptica*-mediated cytotoxicity of macrophages is dependent on *bvg*-regulated factors, including pertactin. Infect. Immun. 67: 5972-5978.
70. Roberts, M. 1999. Vaccination –can you swallow it?. p49 –50. In: The Association of Clinical Pathologists Handbook 1999.
71. Parham, N., Roberts, M. and Williams, A. 2000. Controlling *E.coli* O157:H7 disease in man. – reducing the risk of exposure. Immunol. News April: 71-72.
72. Bacon, A., Makin, J., Sizer, P.J., Jabbal-Gill, I., Hinchcliffe, M., Illum, L., Chatfield, S.N. and Roberts, M. 2000. Carbohydrate Biopolymers Enhance Antibody Responses To Mucosally Delivered Vaccine Antigens. 68: Infect. Immun. 5764-5770.
73. Roberts, M., Chatfield, S., Pickard, D., Li, J., and Bacon, A. 2000. Comparison of the ability of *S.typhimurium aroA aroD* and *aroA htrA* mutants to act as live vectors. Infect. Immun. 68: 6041-6043.
74. Everest, P., Wain, J., Roberts, M., Rook, G and Dougan, G. 2001. The molecular mechanisms of severe typhoid fever. Trends. Microbiol. 9: 316-320.
75. Roberts, M. and Parton, R. 2001. *Bordetella pertussis*. Chp75 pp 1565 - 1619 in "Molecular Medical Microbiology". Ed M.Sussman, Academic Press, London.
76. Spencer, J., Parham, N., Roberts, M., Taylor, D. and Williams, A. 2001. Adherence mechanisms of human diarrhoeagenic *Escherichia coli* and carriage of *E.coli* O157 in bovines. Res. Vet.Sci. 70 sup A: 11.
77. Parham, N., Spencer, J., Roberts, M., Taylor, D. and Williams, A. 2001. The use of immunomagnetic cell separation for isolation of *Escherichia coli* O157:H7. Res. Vet.Sci. 70 sup A: 11.
78. Kenyon, W.J., Sayers, G., Humphreys, S., Roberts, M. and Spector, M.P. 2002. The *rpoE*-Encoded Extracytoplasmic Function Sigma Factor σ^E is Another Sigma Factor

- Required for the Starvation-Stress Response of *Salmonella enterica* serovar Typhimurium. *Microbiology*. 148:113-22.
79. Stevenson A, Roberts M. 2002. Use of a rationally attenuated *Bordetella bronchiseptica* as a live mucosal vaccine and vector for heterologous antigens. *Vaccine* 20:2325-35.
80. Stevenson A, MacDonald, J. and Roberts M. 2003. Cloning and characterisation of type 4 fimbrial genes from *Actinobacillus pleuropneumoniae*. *Veterinary Microbiology*. 92: 121-134.
81. Stevenson A, Roberts M. 2002. Use of *Bordetella bronchiseptica* and *Bordetella pertussis* as live vaccines and vectors for heterologous antigens. *FEMS Immunol. Med. Microbiol.*, 37:121-128.
82. *Immunology and Medical Microbiology*. Accepted for publication.
83. Parham, N., Spencer, J., Taylor, D., Roberts, M., Tement, H., Innocent, G., Mellor, D. and Williams, A. 2003. An adapted ImmunoMagnetic cell Separation (IMS) method for use in quantification of *Escherichia coli* O157:H7 from bovine faeces. *Journal of Microbiological Methods*. 53:1-9
84. Humphreys, S., Rowley, G., Stevenson, A., Kenyon, W.J., Spector, M.P., and Roberts, M. (2003): Role of Periplasmic Peptidylprolyl Isomerases in *Salmonella enterica* Serovar Typhimurium Virulence. *Infect. Immun.*, 71:5386-5388.
85. Miticka, H., Rowley, G., Rezuchova, B., Homerova, D., Humphreys, S., Farn, J., Roberts, M., and Kormanec, J. (2003): Transcriptional analysis of the *rpoE* gene encoding extracytoplasmic stress response sigma factor sigmaE in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol. Lett.*, 226:307-314.
86. Rezuchova, B., Miticka, H., Homerova, D., Roberts, M., and Kormanec, J. (2003): New members of the *Escherichia coli* [sigma]E regulon identified by a two-plasmid system. *FEMS Microbiology Letters*, 225:1-7.
87. Stevenson, A. and Roberts, M. (2003): Use of *Bordetella bronchiseptica* and *Bordetella pertussis* as live vaccines and vectors for heterologous antigens. *FEMS Immunol. Med. Microbiol.*, 37:121-128.
88. Humphreys, S., Rowley, G., Stevenson, A., Anjum, M.F., Woodward, M.J., Gilbert, S., Kormanec, J., and Roberts, M. (2004): Role of the two-component regulator CpxAR in the virulence of *Salmonella enterica* serotype Typhimurium. *Infect Immun*, 72:4654-4661.
89. Miticka, H., Rezuchova, B., Homerova, D., Roberts, M., and Kormanec, J. (2004): Identification of nucleotides critical for activity of the sigma(E)-dependent *rpoE*_{p3} promoter in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol. Lett.*, 238:227-233.

90. Stevenson,A. and Roberts,M. (2004): Intranasal immunisation against tetanus with an attenuated *Bordetella bronchiseptica* vector expressing FrgC: improved immunogenicity using a Bvg-regulated promoter to express FrgC. *Vaccine*, In press:

R. Goringe

ADP-ribosyltransferase. *J. Biol. Chem.*, 258, 185a). *Pertussis Toxin*. Academic Press,

M. J. (1985b). Pertussis toxin: Structure and cells. In *Pertussis Toxin* (Eds R. D. A. Academic Press, New York. A simple chemically defined medium for *J. Gen. Microbiol.*, 63, 211-220. s, M., and Christophe, J. (1986). Rapidly determining affinity and hydrophobic chro-

, M., Ito, K., Katada, T., Ui, M. and t-activating protein, pertussis toxin, in 21, 5516-5522. Islet-activating protein, pertussis toxin: Multiple biological actions. In *Pertussis Toxin* (Eds R. D. A. Academic Press, New

a unit process. In *Progress in Industrial Microbiology*, pp. 151-271. Churchill Livingstone,

Enzyme-linked immunosorbent assay. Rose and H. Friedman), pp. 359-371.

Islet-activating protein. *Pharmac. Ther.*, 19,

pertussigen from *Bordetella pertussis* of strain 28, 509-515. Analysis of phase change in *Bordetella*

amura, T., Nogimori, K., Mizushima, T., and Ito, K. (1985). Islet-activating protein (IAP) in *Bordetella pertussis* of rats. *J. Biochem.*, 83, 295-303. amura, T., Takahashi, I., and Ui, M. (1986). Islet-activating protein (IAP) purified from the strain 28, 305-312.

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CHAPTER 6

The Multiple Biological Activities of Pertussis Toxin

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1. INTRODUCTION

Bordetella pertussis has long been known to produce an exotoxin (or toxins) exhibiting diverse biological activities including those of a lymphocytosis-promoting factor (LPF), a histamine-sensitizing factor (HSF), a haemagglutinin (HA), a mouse-protective antigen (MPA), an adjuvant and a mitogen. An additional activity, found later, was inhibition of adrenaline-induced hyperglycaemia as observed when the catecholamine was injected into rats previously inoculated intraperitoneally (ip) with pertussis vaccine (see Ui, 1984; Ui *et al.*, 1984a, b for review). This unique action of pertussis vaccine was explained by Sumi and Ui (1975) as resulting from the marked hyperinsulinaemia induced by adrenaline in the vaccine-treated animal; thus the hyperglycaemia, which was otherwise seen after adrenaline injection, was effectively antagonized by the unusually large amounts of insulin released in response to the catecholamine (Katada and Ui, 1976). The hyperinsulinaemia resulted from increased insulin secretion from the pancreatic islets of the vaccine-treated rats (Katada and Ui, 1977, 1979a,b). Thus, the activity of pertussis toxin as an islet-activating protein (IAP) was added to the list of its diverse biological activities (Yajima *et al.*, 1978a,b).

IAP was purified from the culture supernate of *B. pertussis* (Tohama strain, phase I) to complete homogeneity (Yajima *et al.*, 1978a). The fully purified protein displayed all the biological activities above described for pertussis toxin(s), i.e. it acted not only as IAP but also as LPF, HSP, HA, MPA, an adjuvant, a mitogen, etc. It is thus remarkable for only one entity to be responsible for such a wide variety of biological activities. The principal purpose of this chapter is to answer the question of how a single protein could be endowed with these multiple functions.

2. THE A-B STRUCTURE OF PERTUSSIS TOXIN

Pertussis toxin (PT) is a hexamer ($M_r = 117\,000$) of five dissimilar subunits which were named in the order of decreasing molecular size: S1 ($M_r = 28\,000$), S2 (23 000), S3 (22 000), S4 (11 700), and S5 (9300). Exposure of the toxin to 5 M urea at 4 °C for 3–4 days gave four separate peaks upon subsequent column chromatography with CM-Sephadex; two of these peaks were due to S1 and S5 and the other two were due to dimers (dimer 1 or D1 and dimer 2 or D2). These two dimers were further split, by exposure to 8 M urea for 16 h followed by DEAE-Sephadex column chromatography, into their constituent subunits: D1 to S2 and S4 and D2 to S3 and S4. Thus, the five subunits were separated from each other and purified to homogeneity, as revealed by individual sharp single peaks on SDS-PAGE (Tamura *et al.*, 1982). This two-step procedure was essential for quantitative resolution of the holotoxin into the constituent subunits, since S5 was too labile to survive the process of exposure to 8 M urea.

CTION

to produce an exotoxin (or toxins) including those of a lymphocytosis-inducing factor (HSF), a haemagglutinin (HA), an adjuvant and a mitogen. An injection of adrenaline-induced hyperglycaemia was injected into rats previously vaccinated (see Ui, 1984; Ui *et al.*, 1982). The hyperglycaemia induced by pertussis vaccine was explained by the marked hyperinsulinaemia induced by the vaccine; thus the hyperglycaemia, which is a biological activity, was effectively antagonized by the vaccine. In response to the catecholamine-induced hyperinsulinaemia resulted from increased insulin secretion in the vaccine-treated rats (Katada and Ui, 1979a,b). The pertussis toxin as an islet-activating protein has biological activities (Yajima *et al.*, 1982).

ernate of *B. pertussis* (Tohama strain, Na *et al.*, 1978a). The fully purified toxin was described for pertussis toxin as also as LPF, HSF, HA, MPA, an adjuvant for only one entity to be a biological activity. The principal purpose of how a single protein could be

OF PERTUSSIS TOXIN

(117 000) of five dissimilar subunits having molecular size: S1 ($M_r = 28\ 000$), S2 ($M_r = 26\ 000$), S3 ($M_r = 26\ 000$), S4 ($M_r = 26\ 000$), and S5 (9300). Exposure of the toxin to separate peaks upon subsequent column chromatography of these peaks were due to S1 and S2 (dimer 1 or D1 and dimer 2 or D2). Exposure to 8 M urea for 16 h followed by separation into their constituent subunits: thus, the five subunits were separated by SDS-PAGE, as revealed by individual sharp bands (Ui *et al.*, 1982). This two-step procedure of the holotoxin into the constituent subunits is the process of exposure to 8 M urea.

Based on the relative colour intensity of the individual subunits stained after SDS-PAGE, the molecular ratio of these subunits in the toxin molecule was calculated as 1 (S1) : 1 (S2) : 1 (S3) : 2 (S4) : 1 (S5).

Reconstitution of the original toxin molecule was next undertaken from these purified subunits. Combination of S2 with S4 or of S3 with S4 in 2 M urea yielded D1 or D2, respectively. No dimer was formed from any other combination. Combination of D1 with D2 failed to form a tetramer, but the further addition of S5, but not S1, to the mixture of D1 and D2 was effective in producing a pentamer, which exhibited no islet-activating activity when injected into rats. S5 is thus referred to as the C subunit because it connects two dimers. The subunit structure and the biological activity of the holotoxin were then recovered by further combination of S1 with the pentamer (Tamura *et al.*, 1982).

The native toxin that had been exposed to urea under milder conditions (5 M urea at 4 °C for only 6 h) was applied to a column of haptoalbumin-Sepharose. A single sharp peak of the protein that passed through the column was identified as S1, while the pentamer was bound to the column and then eluted by 0.5 M NaCl plus 3 M KSCN, again as a sharp peak. Thus, PT was readily dissociated to S1 and the pentamer. S1 was enzymatically active as shown below. Hence, this subunit should be referred to as an A(Active)-protomer. The pentamer appeared to be a B(Binding)-oligomer, because the interaction of PT with haptoalbumin, a sialoprotein, was considered by Irons and MacLennan (1979) to afford a model system for the toxin binding to the target cell surface. We have thus proposed the A-B structure of PT (Tamura *et al.*, 1982).

The genes coding for these five subunits of PT were cloned and sequenced by Loch and Keith (1986) and Nicosia *et al.* (1986). The genes are clustered within 3.2 kilobases in the order of S1, S2, S4, S5, and S3. All subunits contain signal peptides of variable length. The molecular weights calculated for the matured subunits were in good agreement with the M_r values estimated above from their mobilities on a SDS-PAGE. Subunits S2 and S3 share 70% amino acid homology. Thus, the two dimers, D1 and D2, must be very closely related to each other in amino acid composition, although their roles in the B-oligomer functions are distinct (Section 6.2).

3. ADP-RIBOSYLTRANSFERASE ACTIVITY OF THE A-PROTOMER OF PT

3.1 Early studies on islet activation by PT

An outline of the early IAP studies is as follows. Cyclic AMP (cAMP) is the second messenger for insulin secretion, which in turn is the target of PT (IAP) in pancreatic islets both *in vivo* and *in vitro* (Katada and Ui, 1979a,b, 1981b). β -Adrenergic stimulation of insulin secretion was attributed to increases in

cAMP in cells reflecting activation of adenylate cyclase, while α -adrenergic inhibition of the secretion resulted from inhibition of the cyclase giving decreased cellular cAMP. For the adenylate cyclase inhibition by α -adrenergic receptors, the receptors were later found to belong to the α_2 -subtype (Yamazaki *et al.*, 1982), and were selectively modified by PT. No inhibition of adenylate cyclase was observed when α_2 -adrenergic receptors were stimulated in islets that had been exposed to a low concentration of the toxin for several hours *in vitro* or in islets from rats that had been injected with a small amount of the toxin several days before (Katada and Ui, 1979a,b, 1981b).

The action of PT was not restricted to the α_2 -adrenergic receptor system in pancreatic islets. Somatostatin-induced decreases in islet cAMP were also abolished by IAP treatment of the cells (Katada and Ui, 1979a, 1981b). Moreover, α_2 -adrenergic, muscarinic, cholinergic, adenosine (A_1) or opiate receptor-mediated decreases in cAMP (or inhibition of adenylate cyclase) were effectively reversed by prior treatment of rat heart cells (Hazeeki and Ui, 1981), rat adipose cells (Murayama and Ui, 1983; Murayama *et al.*, 1983), or NG108-15 cells (Kurose *et al.*, 1983) with PT. Thus, the receptors for which stimulation causes inhibition, rather than activation, of adenylate cyclase proved to be the target of PT in a variety of cells (Ui *et al.*, 1984a-c).

Although the receptor (target of PT) was solely localized in plasma membranes (Katada and Ui, 1981a; Katada *et al.*, 1982), earlier attempts to obtain direct effects of the toxin by itself on membranes were unsuccessful. The addition of NAD (together with ATP) was soon found to be a prerequisite for the toxin to exert its direct influence on isolated membranes (Katada and Ui, 1982a,b).

These findings led to our discovery of ADP-ribosyltransferase activity of PT as will be described next.

3.2 ADP-ribosyltransferase and NAD-glycohydrolase activities

The reason why NAD is indispensable for development of the direct action of PT on cell membranes was studied by the use of NAD differentially radiolabelled at various sites in the molecule (Katada and Ui, 1982a,b). Membranes of rat C6 glioma cells were first incubated with the radioactive NAD in the presence of PT and then dissolved in SDS to be further analysed for radioactive proteins by SDS-PAGE. A protein with the M_r value of 41 000 was labelled when membranes were incubated with NAD in which the adenine, α -P of the ADP or either of the two ribose moieties was radioactive. No radioactivity was incorporated, however, into any membrane protein if [^{14}C -nicotinamide]NAD was used. When membranes were first labelled with [α - ^{32}P]NAD and then incubated with snake venom phosphodiesterase, the radioactivity once incorporated into the $M_r = 41\ 000$ protein was released as 5'-AMP. Thus, PT catalysed

lenylate cyclase, while α -adrenergic inhibition of the cyclase giving the cyclase inhibition by α -adrenergic belong to the α_2 -subtype (Yamazaki and Ui, 1979a). No inhibition of adenylate cyclase was stimulated in islets of the toxin for several hours in injected with a small amount of the toxin (Katada and Ui, 1979a,b, 1981b).

In the α_2 -adrenergic receptor system, decreases in islet cAMP were also observed (Katada and Ui, 1979a, 1981b). Moreover, adenosine (A_1) or opiate receptor-adenylate cyclase were effectively inhibited (Haseki and Ui, 1981), rat adipose cells (Haseki and Ui, 1981), rat adipose cells (Haseki and Ui, 1981), or NG108-15 cells (Haseki and Ui, 1981), receptors for which stimulation causes adenylate cyclase proved to be the target (Haseki and Ui, 1981).

It was solely localized in plasma membrane (Katada and Ui, 1982), earlier attempts to find on membranes were unsuccessful. It was soon found to be a prerequisite on isolated membranes (Katada and

ADP-ribosyltransferase activity of PT

ADP-ribosyltransferase activity of PT

development of the direct action of use of NAD differentially radiolabelled (Katada and Ui, 1982a,b). Membranes of C6 cells with the radioactive NAD in the presence of the toxin were further analysed for radioactive AD in which the adenine, α -P of the AD was radioactive. No radioactivity was observed in the protein if [14 C-nicotinamide]NAD was used. When the AD was radiolabelled with [α - 32 P]NAD and then analysed, the radioactivity once incorporated as 5'-AMP. Thus, PT catalysed

the transfer of the ADP-ribosyl moiety of NAD to, or ADP-ribosylation of, the membrane protein of M_r 41 000.

The ADP-ribosyltransferase also catalysed the hydrolysis of NAD (NAD-glycohydrolase activity) at a detectable, though low, rate in the absence of an appropriate acceptor such as membrane protein. In fact, the S1 subunit displayed NAD-glycohydrolase activity in the absence of membranes, but only if disulphide bonds in the S1 peptide had been cleaved by prior incubation with dithiothreitol (Katada *et al.*, 1983). Thus, S1 is the A-protomer in the sense that it is the Active component of the toxin. ADP-ribosylation of C6 cell membranes was, however, caused by the holotoxin itself as well as the isolated S1 subunit as described above. In this case, ADP-ribosylation occurred even in the absence of dithiothreitol but was markedly reduced by oxidized glutathione. ATP was essential for the holotoxin, but not for the isolated A-protomer, to display ADP-ribosyltransferase or NAD-glycohydrolase activity.

Taken together, these results indicate that PT must necessarily undergo an intracellular processing to yield an active enzyme molecule. The processing may involve an ATP-induced release of the S1 subunit which is then reduced by an intracellular oxidoreductase to be further converted to an active ADP-ribosyltransferase. The processing enzyme was localized in, or firmly bound to, membrane preparations from C6 cells, but was missing in the preparations from rat heart (Kurose and Ui, 1983) or pancreas. Therefore, we have had to use the A-protomer preactivated with dithiothreitol, or the holotoxin preactivated by ATP plus dithiothreitol, in experiments with these latter membrane preparations.

3.3 Characterization of the target protein

ADP-ribosylation of the membrane protein of M_r 41 000 by (the A-protomer of) PT was investigated as follows (Ui *et al.*, 1985a; 1986a).

1. C6 cell membranes were first incubated with [α - 32 P]NAD and PT and then incubated with trypsin to cause hydrolysis of membrane proteins including the ADP-ribosylated M_r = 41 000 protein. The labelled proteins were separated by SDS-PAGE. The tryptic digestion pattern of the ADP-ribosylated protein was profoundly affected by non-hydrolysable GTP analogues (Gpp(NH)p and GTP γ S) or NaF added simultaneously with the trypsin. Thus, the M_r = 41 000 protein is a GTP-binding protein to which fluoride ions also bind selectively.
2. Incubation of C6 cell membranes with [α - 32 P]NAD and the A_1 -subunit of cholera toxin resulted in ADP-ribosylation of membrane proteins of M_r = 45 000 and 52 000 which were distinctly different from the PT substrate protein of M_r 41 000. The cholera toxin substrates also proved to be GTP-binding proteins.

binding proteins, because their susceptibility to tryptic digestion was influenced by GTP analogues.

3. The ADP-ribosylation of membrane proteins in the presence of the A-protomer of PT and [α - 32 P]NAD was compared between membranes from cells (toxin-treated cells) which had been exposed to PT and those from cells (control cells) that had not been exposed to the toxin. Much less radioactivity was detected in the $M_r = 41\ 000$ protein in membranes from toxin-treated cells than in membranes from control cells. This reflects the previous ADP-ribosylation of the same protein with intracellular non-radioactive NAD during incubation of intact cells with PT. Thus, PT is capable of ADP-ribosylating the membrane protein even when it is added to intact cell preparations and it acts *in vivo*.
4. Some membrane receptors are coupled to adenylate cyclase in an inhibitory fashion, i.e. stimulation of these receptors by agonists causes decreases in cAMP in cells as a result of the inhibition of adenylate cyclase, and this inhibition is observable in membranes prepared therefrom. In cells exposed to increasing concentrations of PT prior to receptor stimulation, the decreases in cAMP (or the inhibition of adenylate cyclase activity in membranes from these cells) caused by the subsequent addition of receptor agonists became smaller as the concentration of the toxin, to which the cells had been exposed, increased. The ADP-ribosylation by PT of the $M_r = 41\ 000$ protein in membranes from these toxin-treated cells also increased progressively, in parallel to the attenuation of adenylate cyclase inhibition, as the concentration of the toxin increased. The degree of ADP-ribosylation occurring in cells in response to pertussis toxin was well correlated with the toxin-induced attenuation of the inhibition of adenylate cyclase. Taking advantage of the strategy developed in (3) above, the ADP-ribosylation of the $M_r = 41\ 000$ protein by PT was shown to be responsible for the toxin-induced reversal of receptor-mediated inhibition of adenylate cyclase in intact cells as well as in isolated membranes.

Thus, the $M_r = 41\ 000$ protein serving as the substrate of PT proved to be the GTP-binding protein (currently referred to as G_i or N_i where the subscript i stands for 'inhibitory') which acts as a transducer to couple receptors to the adenylate cyclase catalytic protein in an inhibitory fashion in membranes. G_i loses its function as the transducer after being selectively ADP-ribosylated by PT. This is exactly the mechanism of action of PT in its rôle as IAP. The protein ADP-ribosylated by cholera toxin proved then to be G_s or N_s (where the subscript s stands for 'stimulatory') which mediates receptor-coupled activation of adenylate cyclase. A proposal has been made for possible ADP-ribosylation of G_i by endogenous ADP-ribosyltransferase in rat liver under certain pathological conditions, though the enzyme still remains to be characterized (Itoh *et al.*, 1984; Ui *et al.*, 1985c).

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4. DUAL FUNCTIONS OF THE B-OLIGOMER OF PT

4.1 A-protomer-transporting activity

The rôle of the B-oligomer moiety in the action of PT was studied by the use of antibodies which selectively interacted with certain domains of the toxin molecule (Tamura *et al.*, 1983). Polyclonal antibodies were raised in rabbits against PT holotoxin, its S1-subunit (A-protomer) and its B-oligomer. The IgG fractions isolated from these antisera interacted with their antigen selectively; the anti-holotoxin IgG bound to both the A-protomer and the B-oligomer of the toxin, while the anti-A-protomer and anti-B-oligomer IgGs immunoprecipitated only with the A-protomer and the B-oligomer, respectively.

C6 cells were exposed to a fixed concentration of PT in the absence or presence of increasing concentrations of these IgG preparations. GTP-dependent adenylate cyclase activity of membranes prepared from the cells exposed to the toxin alone was greater than the activity of membranes from the control cells (not exposed to the toxin) due to ADP-ribosylation of the membrane G_i protein. These effects of PT were suppressed progressively when the concentration of antibodies simultaneously added was increased. The anti-B-oligomer IgG was as effective as the anti-A-protomer IgG in this regard, whereas normal serum IgG failed to inhibit the actions of the toxin.

Direct action of PT on isolated C6 cell membranes was next studied in the presence of NAD and ATP. Simultaneous addition of the anti-A-protomer IgG interfered with the toxin-induced increases in the GTP-dependent adenylate cyclase activity and ADP-ribosylation of the membrane G_i . In contrast, no interference was observed with the anti-B-oligomer IgG. Thus, the B-oligomer moiety plays an indispensable rôle in the interaction of PT with intact cells, but is not required for its direct interaction with isolated membranes.

The actions of PT to increase GTP-dependent adenylate cyclase and to induce ADP-ribosylation of G_i in intact C6 cells were antagonized by the isolated B-oligomer, which by itself was without effect in these cells. The antagonism was 'competitive' in nature; i.e. the dose-response curves for the holotoxin were shifted to the right by the isolated B-oligomer simultaneously added in a single dose. The B-oligomer is likely to occupy the same sites as the holotoxin on the cell surface. Thus, the indispensable rôle of the B-oligomer in the action of PT on intact cells can be readily accounted for by the binding of the holotoxin through its B-oligomer moiety. The B-oligomer probably binds to particular glycoproteins on the cell surface as the first step of the interaction of PT with intact cells. The slow internalization of the membrane glycoproteins may then afford a means for the toxin to traverse the plasma membrane eventually reaching the cytosolic side where a processing occurs (as described in Section 3.2) to convert the toxin to its active form of ADP-ribosyltransferase. The definite lag time necessarily preceding the onset of the action of PT on intact

cells (Katada and Ui, 1980) is very likely to reflect the time of this internalization of the toxin molecule because the lag time became longer as the temperature was lowered to slow down the internalization rate (Katada and Ui, 1980).

4.2 Mitogenic action

The addition of PT to mouse or rat splenic cells increased the incorporation of [3 H]thymidine into DNA fractions in these cells (Tamura *et al.*, 1983). This mitogenic action of the holotoxin was reproduced by the isolated B-oligomer of the toxin; it was due to binding of the B-oligomer to the cells. The B-oligomer was as active as, or occasionally even more active than, the holotoxin as mitogen. There was no effect seen in the splenic cells from thymus-deficient nude mice. Thus, the B-oligomer of PT is a potent T-cell mitogen. The B-oligomer was bound to the T-cell surface via its two constituent dimers, D1 and D2, as evidenced by competitive inhibition of the B-oligomer-induced mitogenesis by either of these two dimers. Neither D1 nor D2 was mitogenic by itself. It is very likely, therefore, that the 'divalent' binding of the B-oligomer to the T-cell surface via two dimers results in cross-linking of glycoproteins leading to T-lymphocyte proliferation.

Cross-linking of membrane proteins by concanavalin A, another T-cell mitogen, is known to cause stimulation of glucose oxidation in adipocytes. Glucose oxidation was stimulated by PT also, but not by D1 or D2, confirming our idea that cross-linking of membrane proteins is responsible for the mitogenic action of the B-oligomer (Tamura *et al.*, 1983). The B-oligomer exhibited no detectable activities *in vivo* when injected into rats, however, probably due to its instability in the circulation.

Thus, the B-oligomer has dual functions: one as a carrier of the A-protomer which is responsible for ADP-ribosylation of proteins inside the membrane of target cells and the other as a mitogen of T-cells (or other cells) in which ADP-ribosylation by the A-protomer is not involved. The following section (Section 5) of this chapter will be devoted to the important question of which of these two functions is involved in each of the multiple actions *in vivo* of PT. The different mechanisms of B-oligomer binding to the cells involved in these two functions will be described in Section 6.

5. MULTIPLE MECHANISMS FOR THE BIOLOGICAL ACTIVITIES OF PT

5.1 Chemical modification of free amino groups

The biological activities *in vivo* and *in vitro* of PT were profoundly affected by chemical modification of free amino groups of the lysine residues in the toxin molecule. After being acylated (acetylated, maleylated or succinylated) the toxin

lect the time of this internalization became longer as the temperature rate (Katada and Ui, 1980).

cells increased the incorporation of cells (Tamura *et al.*, 1983). This induced by the isolated B-oligomer B-oligomer to the cells. The B-oligomer is more active than, the holotoxin in splenic cells from thymus-deficient mice. The B-oligomer is a potent T-cell mitogen. The B-oligomer consists of two constituent dimers, D1 and D2. The B-oligomer-induced mitogenic activity of either D1 nor D2 was mitogenic. The 'divalent' binding of the B-oligomer results in cross-linking of glycoproteins.

Concanavalin A, another T-cell lectin, induced glucose oxidation in adipocytes, but not by D1 or D2, confirming that the B-oligomer is responsible for the mitogenic activity (1983). The B-oligomer exhibited mitogenic activity in rats, however, probably due

to its acting as a carrier of the A-protomer proteins inside the membrane of cells (or other cells) in which ADP-ribosylation occurs. The following section (Section 5.2) discusses the question of which of these multiple actions *in vivo* of PT. The cells involved in these two

5. BIOLOGICAL ACTIVITIES

5.1

PT were profoundly affected by the modification of the lysine residues in the toxin molecule (acylated or succinylated) the toxin

exhibited no biological activity *in vivo*, because of destruction of its quaternary structure (Nogimori *et al.*, 1984a). Acetamidation was a milder modification of the same amino groups; the subunit assembly of the toxin was maintained after the exhaustive (80–90%) acetamidation of lysine residues, as revealed by unaltered patterns of gel filtration and disc electrophoresis (Ui *et al.*, 1985b). None of the other amino acid residues was modified in the toxin molecule under the same conditions.

Acetamidation of PT was therefore promising as a means of differential modification of biological activities of the toxin. The toxin fully acetamidated acted as an IAP, equally potent and active as the native (unmodified) toxin, i.e. as much insulin was secreted in the acetamidated toxin-injected rats as in the native toxin-injected rats in response to glucose load. The IAP action of PT is solely dependent on ADP-ribosylation of G_i in islet cells by the A-protomer which has been transported into the cells by means of the B-oligomer binding. Thus, acetamidation of the toxin molecule did not interfere with functions essential for the development of the IAP action. These functions are related to: (1) the binding of the B-oligomer to the target cell surface to transport the toxin into the cells; (2) the susceptibility of the toxin thus transported to intracellular processing; and (3) the ADP-ribosyltransferase activity of the A-protomer after being processed. In sharp contrast, neither the holotoxin nor the B-oligomer caused mitosis after these proteins had been acetamidated. Thus, ε-amino groups of the lysine residues should be maintained unmodified for the B-oligomer to bind 'divalently' to the membrane glycoproteins for mitogenic activity.

5.2 Classification of biological activities of PT by their susceptibility to acetamidation of lysine residues

Diverse biological activities *in vivo* and *in vitro* of PT were studied either before or after acetamidation of the toxin. The activities could be segregated into four classes (Class 1 to Class 4, Table 1) which showed different susceptibilities to acetamidation (Nogimori *et al.*, 1984b; 1986a) and which presumably reflect different mechanisms of action.

5.2.1 Activities not impaired by acetamidation: 'Class 1' activities

Acetamidated PT was as effective as the native toxin in causing ADP-ribosylation of membrane G_i when isolated membranes were incubated with these toxins in the presence of NAD (1.a, Table 1). Thus, the foregoing statements ((2) and (3) in Section 5.1) were confirmed, i.e. the processing of the holotoxin to yield an active ADP-ribosyltransferase, together with this enzymic activity itself, was not impaired by acetamidation of the toxin molecule.

The 'Class 1' effects of PT *in vitro* on intact cells (1.b,c,d, Table 1) also were

Table 1 Classification of biological activities of pertussis toxin

Class	Biological activities	Attenuation by acetamidination
1	(a) ADP-ribosylation of G-proteins	No
	(b) Potentiation of adenylate cyclase	No
	(c) Stimulation of insulin secretion	No
	(d) Stimulation of glycerol release	No
	(e) Inhibition of adrenaline hyperglycaemia	No
	(f) Potentiation of hyperinsulinaemia (IAP activity)	No
	(g) Hypotensive activity	No
	(h) Positive inotropic activity	No
2	(a) Mitogenic activity	Yes
	(b) Stimulation of glucose oxidation	Yes
	(c) Promotion of lymphocytosis	Yes
	(d) Increase in vascular permeability	Yes
	(e) Histamine-sensitizing activity	Yes
	(f) Adjuvant activity	Yes
3	Inhibition of histamine release	Yes
4	Haemagglutinin activity	No

not influenced by acetamidination. These effects are due to ADP-ribosylation of G_i in these cells; G_i , upon being ADP-ribosylated, loses its function to mediate receptor-linked inhibition of adenylate cyclase. The typical receptors involved in the inhibition include α_2 -adrenergic and A_1 -adenosine receptors. The loss of the G_i function is involved in increased GTP-dependent adenylate cyclase activity in membranes from toxin-treated C6 cells (Katada *et al.*, 1982). The cyclase activity increased to the same level whether the cells had been exposed to the native or acetamidinated toxin (1.b). The G_i -mediated inhibition of adenylate cyclase is responsible for the inhibition of insulin secretion from isolated pancreatic islets observable upon the addition of adrenaline (an α_2 -adrenergic agonist) to the cells and in the inhibition of glycerol release from adipocytes in the inevitable presence of endogenous adenosine. Since the G_i -mediated inhibition was reversed by PT, more insulin or more glycerol was released from islets (1.c) or adipocytes (1.d), respectively, with PT in the pre-incubation medium than in its absence. The acetamidinated toxin was as effective as the native toxin in this regard. PT has to enter the cells by virtue of the B-oligomer binding to the cell surface before G_i is ADP-ribosylated in intact cells. Thus, the equal effectiveness of the acetamidinated toxin to the native toxin on intact cells supports the foregoing notion ((1) in Section 5.1) that no free amino groups are required for the binding of the B-oligomer to the target cell surface to translocate the toxin into the cell.

Some of the actions *in vivo* of PT survived the acetamidination process

al activities of pertussis toxin

	Attenuation by acetamidination
	No
	No
	No
	No
caemia	No
a (IAP activity)	No
	No
	Yes
	Yes
	Yes
	Yes
	Yes
	Yes
	Yes
	No

effects are due to ADP-ribosylation of G_i or other toxin-sensitive GTP-binding proteins (see Section 7 below) in target cells. They include hypotension (1.g) and positive inotropism (1.h) (and chronotropism) observed for 1-2 weeks following a single injection of the native or acetamidinated toxin into spontaneously hypertensive rats. Enhanced β -adrenergic responses to endogenous catecholamines are in all likelihood involved in these cardiovascular effects of the toxin, since the effects were antagonized by β -adrenergic antagonists. Probably, the function of G_i negatively coupled to cardiovascular β -adrenergic receptors (Murayama and Ui, 1983; Ui *et al.*, 1984d) was abolished by the toxin-catalysed ADP-ribosylation. Thus, these actions of PT *in vivo*, together with the adrenaline-hyperglycaemia-inhibitory (1.e) and hyperinsulinaemia-inducing (IAP, 1.f) actions, came into the same category of 'Class 1' in Table 1.

arrived the acetamidination process

(Nogimori *et al.*, 1984b; 1.e,f,g,h, Table 1). These actions must result from ADP-ribosylation of G_i or other toxin-sensitive GTP-binding proteins (see Section 7 below) in target cells. They include hypotension (1.g) and positive inotropism (1.h) (and chronotropism) observed for 1-2 weeks following a single injection of the native or acetamidinated toxin into spontaneously hypertensive rats. Enhanced β -adrenergic responses to endogenous catecholamines are in all likelihood involved in these cardiovascular effects of the toxin, since the effects were antagonized by β -adrenergic antagonists. Probably, the function of G_i negatively coupled to cardiovascular β -adrenergic receptors (Murayama and Ui, 1983; Ui *et al.*, 1984d) was abolished by the toxin-catalysed ADP-ribosylation. Thus, these actions of PT *in vivo*, together with the adrenaline-hyperglycaemia-inhibitory (1.e) and hyperinsulinaemia-inducing (IAP, 1.f) actions, came into the same category of 'Class 1' in Table 1.

5.2.2 Activities abolished by acetamidination: 'Class 2' activities

The foregoing idea (see Section 5.1) that unmodified lysine residues are essential for the B-oligomer to bind to cells in a divalent manner was confirmed by marked attenuation of the insulin-like action of PT on adipocytes which also depends on cross-linking of membrane proteins caused by divalently bound toxin molecules (2.b, Table 1). The same actions of the isolated B-oligomer on lymphocytes and adipocytes were also suppressed by acetamidination of the protein. Thus, we propose that the biological activities of PT that were severely impaired by acetamidination of the toxin molecule result from stimulation of cells due to divalent binding of the B-oligomer to membrane proteins. These activities including lymphocytosis-promoting (2.c, Table 1), histamine-sensitizing (2.e) and adjuvant (2.f) activities of pertussis toxin are listed under 'Class 2' in Table 1.

The 'Class 2' activities were also distinct from the 'Class 1' activities in that much higher concentrations of PT were required for development of activities. Large amounts of the B-oligomer are probably needed for cross-linking of membrane proteins, whereas ADP-ribosylation proceeds in a manner catalytically dependent on a very few molecules of the A-protomer that have been internalized by the aid of the B-oligomer at the 1:1 molar ratio.

The widely known action of PT to promote lymphocytosis depends on its direct action on lymphocytes (Sugimoto *et al.*, 1983), in which the same mechanisms are involved as in the mitogenic action of the toxin (2.a). Stimulation of lymphocytes would trigger certain immune reactions leading to facilitated antibody formation (the adjuvant activity). Moreover, the endothelial cells of pulmonary vessels would contract and shrink, upon stimulation by PT, permitting outward passage of plasma proteins and fluid into the extracellular spaces (the increase in vascular permeability, 2.d in Table 1). This would be respon-

sible for the toxin-induced increase in histamine death (the activity as HSF). The injection of histamine into the toxin-treated rodents results in hyperinsulinaemia and hence hypoglycaemia by the same mechanism as for 'Class 1' activities (Yajima *et al.*, 1981). Although this hypoglycaemia is partly responsible for the toxin-induced enhancement of histamine death, the increased vascular permeability is more important, since the activity of the toxin as HSF was still observed even when hypoglycaemia was prevented by glucose infusion into the animal. Since cAMP is inhibitory, rather than stimulatory, to the cellular responses that play essential rôles in development of immune or inflammatory reactions, it is unlikely that the A-protomer which acts to increase the cellular cAMP content is involved in these 'Class 2' activities of PT.

5.2.3 Other possible mechanisms in certain biological activities of PT

Induction of histamine release from rat mast cells by compound 48/80 was strongly inhibited by prior exposure of the cells to PT (Nakamura and Ui, 1983). The potency of the toxin to inhibit the histamine secretion was markedly diminished by acetamidination of the lysine residues in the toxin molecule (Nogimori *et al.*, 1984b), indicating that the activity should fall into the category of 'Class 2' in Table 1. Our recent study has shown, however, that the toxin-induced inhibition of histamine secretion, just like the 'Class 1' activities of the toxin, arose from ADP-ribosylation of GTP-binding proteins by the A-protomer of PT (Nakamura and Ui, 1984, 1985). This action of PT was not reproduced by its B-oligomer separated from the A-protomer. Furthermore, the concentrations of PT required to cause this action were much lower than those required for other 'Class 2' activities. Thus, the mechanism for this toxin activity may belong to 'Class 1' while its susceptibility to acetamidination makes it reasonable to bring this activity under the category of 'Class 2'. It is therefore classified as 'Class 3' in Table 1.

Mast cells, or basophils, originate from the haematopoietic stem cells and are involved in inflammatory and immune responses. The B-oligomer of PT might bind to the surface of these blood cells in a manner somewhat different from its binding to non-blood cells such as pancreatic islets. This type of binding might be unique in depending on the lysine residues which are acetamidinated under the conditions employed in the present study.

Neither a monomer nor a dimer that constitutes the B-oligomer moiety of PT exhibited, by itself, any of the biological activities discussed above. The haemagglutinin activity was an exception; D1 was as effective as the native toxin in this regard (Nogimori *et al.*, 1986a). Thus, the mechanism for the haemagglutinin activity of PT must be distinct from the mechanism for either the 'Class 1' or 'Class 2' activities. It is therefore placed under 'Class 4' in Table 1. This activity was not affected by acetamidination of the lysine residues in the peptides.

stimine death (the activity as HSF). treated rodents results in hyperinsulin-
me mechanism as for 'Class 1' activi-
hypoglycaemia is partly responsible
amine death, the increased vascular
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has shown, however, that the toxin-
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'-binding proteins by the A-protomer
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protomer. Furthermore, the concen-
were much lower than those required
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6. DUAL MANNER OF TOXIN BINDING TO TARGET CELLS AS REVEALED BY HYBRID TOXINS

6.1 Preparation of hybrid toxins

The foregoing results (Section 5) obtained with acetamidinated PT revealed that two major mechanisms are involved in the diverse biological activities of the toxin. The relative rôles of constituent subunits in these differential mechanisms have been further studied with hybrid toxins in which particular subunits were selectively modified and others left unaltered (Nogimori *et al.*, 1986b).

Reductive methylation was adopted, instead of acetamidination, for this purpose, since methylation proceeds irreversibly under milder conditions (Nogimori *et al.*, 1986b). Thus, the reductive methylation of PT was done by exposure of the protein to 10 mM formaldehyde in 15 mM pyridine borane, a mild reducing agent, at pH 7.0 for 2 h in an atmosphere of nitrogen. Over 90% of the amino groups of the lysine residues in the toxin molecule were dimethylated but none of the other constituent amino acids, including cysteine, was modified under these conditions. The resultant methylated PT exhibited the same biological activities as did the acetamidinated toxin; it was as effective as the native toxin in inducing the 'Class 1' and 'Class 4' activities but did not exhibit the 'Class 2' and 'Class 3' activities (see Table 1).

The methylated toxin was resolved into the A-protomer and B-oligomer by maintaining the toxin solution in 4 M urea for 6 h at 4 °C. The A-protomer was adsorbed by a column of DEAE-Sepharose and then eluted therefrom as a single peak with a linear gradient of 0–0.5 M NaCl. The methylated B-oligomer that passed through the column was further resolved into D1, D2, and S5 by exposure to 5 M urea for 24 h at 4 °C. These methylated monomers and dimers were purified by application of the urea solution to a column of CM-Sepharose. Each of them gave a sharp single band on disc electrophoresis with the same migration rate as the corresponding native protein. These methylated components were then cross-combined with the components derived from the native toxin to afford four kinds of hybrid toxins with compositions and yields as shown in Table 2.

6.2 Essential rôle of the lysine free amino groups in dimer-2 binding

Hybrid toxins thus prepared as shown in Table 2 were analysed for their biological activities. All the hybrid toxins tested, as well as the methylated PT, caused the same degree of glycerol release as did the native toxin in a similar dose-dependent manner with concentrations from 1 to 200 ng/ml. As mentioned above, stimulation of glycerol release from adipocytes, like insulin release from islets, is one of the assay systems for ADP-ribosylation of G_i by the A-protomer transported and processed after the binding of PT via the B-oligomer to the

Table 2 Compositions, yields, and mitogenic activities of hybrid toxins

Abbreviations*	Methylation of components				Yield (%)	Mitogenic activity ^b
	A-protomer	D1	D2	C-subunit		
H-AC	yes	no	no	yes	45.8	+
H-D1	no	yes	no	no	42.9	+
H-D2	no	no	yes	no	57.6	-
H-DD	no	yes	yes	no	30.1	-

* Abbreviations of hybrid toxins are such that H (hybrid) is followed by the components that are methylated. A, C, D1, D2, and DD represent the A-protomer, C-subunit (S5), dimer 1 (D1), dimer 2 (D2), and both dimers, respectively.

^b The mitogenic activity of hybrid toxins at 2-4 µg/ml is listed: (+) active; (-) inactive.

cells. Thus, the previous conclusion (Section 5.2.1) was again confirmed that the free amino groups in peptides are not essential for the A-protomer-transporting activity of the B-oligomer or for the development of the ADP-ribosyltransferase activity of the A-protomer of PT. In fact, other 'Class 1' activities of PT were also reproduced by all the hybrid toxins.

Of more interest were the 'Class 2' activities of hybrid toxins, since these activities of the native toxin were not mimicked by the acetamidinated or methylated toxin. The hybrid toxins in which D2 was not methylated (H-AC and H-D1) were as effective as the native PT, while the toxins possessing methylated D2 (H-D2 and H-DD) were essentially without effect, in eliciting mitosis of lymphocytes. Thus, the methylation of D2 did, but the methylation of D1 or S5 did not, interfere with the mitogenicity of the B-oligomer. It is very likely that the free amino groups in D2 play an important rôle in binding of the B-oligomer to stimulate lymphocytes.

The same influences were exerted by hybrid toxins upon the number of circulating leukocytes *in vivo*. H-AC and H-D1 were as effective as the native toxin; the action of PT as a LPF was not impaired by methylation of subunits other than D2. In sharp contrast, H-D2 and H-DD were much less effective than the native toxin; the free amino groups in D2 must be required for the LPF activity to occur *in vivo*. These results obtained with hybrid toxins thus give strong support to the foregoing idea (Section 5.2.2) that divalent binding of the B-oligomer to cause mitosis of lymphocytes is responsible for promotion of leukocytosis and other 'Class 2' activities of PT.

6.3 Differential binding of the B-oligomer to target cells

The binding of PT via its B-oligomer moiety to the cell surface is the first step for either the 'Class 1' or 'Class 2' activities to develop *in vivo* or *in vitro*. The rôle of the two dimers in binding was further studied by the dimer-induced competitive inhibition of the actions of native PT *in vitro*.

7i

Mitogenic activities of hybrid toxins

Components	Yield (%)	Mitogenic activity ^a
C-subunit		
yes	45.8	+
no	42.9	+
no	57.6	-
no	30.1	-

hybrid) is followed by the components that the A-protomer, C-subunit (S5), dimer 1

^aml is listed: (+) active; (-) inactive.

on 5.2.1) was again confirmed that the trial for the A-protomer-transporting component of the ADP-ribosyltransferase, other 'Class 1' activities of PT were

activities of hybrid toxins, since these mimicked by the acetamidinated or which D2 was not methylated (H-AC ive PT, while the toxins possessing essentially without effect, in eliciting lation of D2 did, but the methylation mitogenicity of the B-oligomer. It is D2 play an important rôle in binding es.

hybrid toxins upon the number of H-D1 were as effective as the native impaired by methylation of subunits and H-DD were much less effective ups in D2 must be required for the lirs obtained with hybrid toxins thus (Section 5.2.2) that divalent binding phocytes is responsible for promotion es of PT.

er to target cells

ety to the cell surface is the first step es to develop *in vivo* or *in vitro*. The urther studied by the dimer-induced itive PT *in vitro*.

6.3.1 Monovalent binding of PT via either dimer 1 or dimer 2 to introduce the A-protomer into cells

The toxin-induced glycerol release from rat adipocytes was chosen as a representative index of 'Class 1' activities. The antagonism by two dimers of this action of the native toxin was characterized as follows: (1) either D1 or D2 caused inhibition, though D1 was more potent and efficient than D2; (2) methylated D1 was an inhibitor as effective as the native (unmodified) D1, whereas methylated D2 no longer acted as an inhibitor.

Thus, free amino groups of the lysine residues are essential for the binding of D2 but not for the binding of D1 to the cell surface. Hence, methylated D2 is not capable of binding to the cell surface of adipocytes. Nevertheless, H-D2 and H-DD, the hybrid toxins in which D2 was methylated, were essentially as effective as the native PT in stimulating glycerol release from adipocytes. It is likely, therefore, that D1 and D2 in the B-oligomer moiety of the toxin occupy the same sites on adipocytes or other cells to introduce the A-protomer moiety into the cells. In other words, the monovalent binding of the B-oligomer via either D1 or D2 to the same sites must be sufficient for the entrance of the associated A-protomer into the cells. The affinity for these sites was higher with D1 than with D2. Hence, lipolytic action of PT would be antagonized by either D1 or D2 as in (1) above, while hybrid toxins in which D1 and/or D2 was methylated were lipolytic agents as potent as the native PT, because methylated D1 was able to bind to the sites on the cells with the same potency as the non-methylated D1.

6.3.2 Divalent binding of PT via dimer 1 and dimer 2 as a trigger of 'Class 2' activities

Mitogenic action of the native PT (or the native B-oligomer) was antagonized by D1 or D2 in a competitive manner with the following characteristics: (1) D2 was four-fold as potent as D1; (2) methylation interfered with the D2-induced inhibition but not with the D1-induced inhibition. Thus, free amino groups were essential for the binding of D2, but not for the binding of D1, to lymphocytes as well as to adipocytes.

The binding of the toxin via D2 was an indispensable process for toxin-induced mitosis, since the hybrid toxins in which D2 was methylated did not act as mitogen (see Section 6.1). The finding that the mitogenic action of PT was antagonized by D1 as well as by D2 thus lends strong support to the foregoing conclusion that divalent binding via two dimers is essential for 'Class 2' activities of the toxin. As has been discussed previously, the divalent binding may lead to cross-linking of membrane glycoproteins which triggers mitosis of lymphocytes, glucose uptake by adipocytes, and probably other 'Class 2' activities.

7. GTP-BINDING PROTEINS AS SPECIFIC TARGETS OF PT

The target molecule of PT, i.e. the specific substrate of the toxin-catalysed ADP-ribosylation, has proved to be a GTP-binding protein (G-protein), which acts as a transducer communicating between receptors and effectors in membranes of a wide variety of mammalian cells. The route of cellular signalling is as follows. The first step is recognition by receptors of extracellular signals such as neurotransmitters, hormones, and autacoids. Effectors are either enzymes producing such intracellular signals as cyclic nucleotides, diacylglycerol and inositol phosphates, or ion channels facilitating transmembrane passage of cations and anions. These intracellular signals and ions trigger eventual cellular responses such as (i) cell mobilization, including muscle contraction and relaxation and chemotaxis, (ii) exocytosis and endocytosis including endocrine and exocrine secretion and phagocytosis, (iii) energy metabolism, (iv) cell proliferation, and (v) cell differentiation.

Thus, G-proteins play a pivotal rôle in the membrane signal transduction system which is responsible for physiological regulation of cellular functions. Once ADP-ribosylated by PT, G-proteins lose their function as transducers (Murayama and Ui, 1984). Hence, blockade by PT of a cellular response to receptor stimulation, if it occurs, affords convincing evidence for an involvement of a G-protein in the signal system leading to this response (Ui, 1984). A number of investigators have taken advantage of this strategy; PT is in wide use as a valuable reagent in several current research fields in the life sciences. The detailed description of the toxin substrate G-proteins is, however, beyond the scope of this chapter. The following is only a brief summary of what is currently known about G-proteins serving as specific substrates of PT-catalysed ADP-ribosylation:

1. Common properties of G-proteins so far discovered to serve as the substrate of the A-protomer of PT are: (i) they are trimers, each composed of α -, β -, and γ -subunits; (ii) the α -subunit possesses a site occupied by GTP (or GDP) and a site ADP-ribosylated by the toxin; (iii) the site which is ADP-ribosylated is a cysteine at the fourth residue from the carboxyl terminus of the α -peptide; (iv) this cysteine is not ADP-ribosylated by the toxin unless the α -subunit is tightly associated with $\beta\gamma$ -subunits to form a trimeric structure; the isolated α -subunit does not serve as the toxin substrate; (v) the β - and γ -subunits are usually indistinguishable among these G-proteins; they appear to be a mixture of two β -subunits (M_r 35 000 and 36 000) and at least three γ -subunits (M_r = 5000-8000). Thus, each of the G-proteins is recognized by its α -subunit only (see below); (vi) there is also a common mechanism by which these G-proteins act as the transducer in various systems of membrane signal transduction. This will be briefly described later.

Some of these toxin-substrate G-proteins have been purified and genes

SPECIFIC TARGETS OF PT

specific substrate of the toxin-catalysed P-binding protein (G-protein), which between receptors and effectors in cells. The route of cellular signalling by receptors of extracellular signals and autacoids. Effectors are either as cyclic nucleotides, diacylglycerol channels facilitating transmembrane intracellular signals and ions trigger cell mobilization, including muscle contraction, (ii) exocytosis and endocytosis and phagocytosis, (iii) energy metabolism and differentiation.

the membrane signal transduction regulation of cellular functions. lose their function as transducers by PT of a cellular response to convincing evidence for an involvement in this response (Ui, 1984). advantage of this strategy; PT is in wide use in research fields in the life sciences. substrate G-proteins is, however, beyond the scope of this review. This is only a brief summary of what is known about specific substrates of PT-catalysed

or discovered to serve as the substrate are trimers, each composed of α -, β -, and γ -subunits. (i) the site occupied by GTP (or the toxin); (ii) the site which is ADP-ribosylated from the carboxyl terminus of ADP-ribosylated by the toxin unless with $\beta\gamma$ -subunits to form a trimeric complex. (iii) not serve as the toxin substrate; (iv) distinguishable among these G-proteins; (v) subunits (M_r 35 000 and 36 000) and 8000). Thus, each of the G-proteins (see below); (vi) there is also a common as act as the transducer in various reactions. This will be briefly described

proteins have been purified and genes

encoding some of the α -peptides have been cloned and analysed for nucleotide sequences (see Ui, 1986 for review). A brief description will be given below for individual proteins.

2. The G-protein first identified as the substrate of PT was G_i , with the α -subunit of M_r 41 000 (see Section 3.3 above). It acts as a transducer coupling receptors to adenylate cyclase in an inhibitory fashion (Ui, 1984). G_i was purified from rabbit liver (Bokoch *et al.*, 1984; Katada *et al.*, 1984a,b,c) and bovine (see Gilman, 1984 for review) and rat (Katada *et al.*, 1986a) brain. Purified G_i was actually coupled to receptors when it was reconstituted into membranes or phospholipid vesicles containing the receptor protein (Haga *et al.*, 1985; Kurose *et al.*, 1986; Asano *et al.*, 1985). The gene coding for the α -subunit of G_i was recently cloned and sequenced from rat glioma C6 cells (Itoh *et al.*, 1986).
3. The G-protein next found to serve as a PT substrate was transducin, the transducer located in the disc membrane of rod outer segments in vertebrate retinal cells. For visual signalling, the receptor is rhodopsin stimulated by photons, and the role of the effector is played by cGMP phosphodiesterase in the disc membrane (Tsuda *et al.*, 1986). The thus decreased cytosolic cGMP is responsible for light-induced hyperpolarization due to closing of sodium channels in the plasma membrane. The α -subunit of transducin, of M_r 39 000, in rod outer segments is ADP-ribosylated by PT in the dark, which retains this G-protein in its inactive GDP-bound $\alpha\beta\gamma$ -trimer form. Visual signals are thus blocked by PT.
4. In addition to G_i , two other PT substrates have been purified from rat brain (Katada *et al.*, 1986a,b, 1987). One is identical with G_o which had been isolated from bovine brain. The M_r of the α -subunit of G_o is 39 000, though it differed distinctly from α of transducin in its immunochemical properties and susceptibility to tryptic digestion. The other is also a trimer with a novel α -subunit of M_r 40 000 (Katada *et al.*, 1987). The α_{40} -subunit was distinguishable from the α -subunits of other G-proteins (i.e. α_{41} of G_i , α_{39} of G_o , and α_{39} of transducin), since it did not interact with the antibodies raised against these α -subunits. The physiological role of G_o and $\alpha_{40}\beta\gamma$ in the central nervous system is unknown.
5. There are a number of recent publications reporting that G-proteins play an important role as the transducer between receptors and phospholipase C in a variety of cell types (Ui *et al.*, 1984c; 1985d). These G-proteins are occasionally referred to as G_p , where the subscript p stands for phospholipase. Some G_p have been identified as the substrate of PT-catalysed ADP-ribosylation, while others were insensitive to the toxin (Murayama and Ui, 1985, 1987a; Kurose and Ui, 1985; see also Ui, 1986 for review). For instance, receptor-mediated histamine secretion from mast cells and superoxide anion (O_2^-) release from neutrophils were blocked by prior exposure of these cells to PT which caused ADP-ribosylation of a membrane

protein with the M_r value of around 40 000, despite the fact that cAMP is not involved in these cellular responses (Okajima and Ui, 1984; Nakamura and Ui, 1984; Okajima *et al.*, 1985). Later studies revealed that the PT substrate coupled receptors to phospholipase C directly in these cell types (Ohta *et al.*, 1985; Nakamura and Ui, 1985; Kikuchi *et al.*, 1986). Likewise, proliferation of 3T3 fibroblasts was suppressed by PT when DNA synthesis was triggered by certain competence factors (Murayama and Ui, 1987b).

No evidence has been provided thus far for or against the idea that the toxin substrate involved in these types of signalling is identical with G_i , G_o or $\alpha_0\beta\gamma$ as mentioned in item (4) above. Recently, a novel G-protein, $\alpha_0\beta\gamma$, has been purified from HL-60 cells that had been differentiated to reticulocytes by means of dimethyl sulphoxide (Oinuma *et al.*, 1987) but its relationship to $\alpha_0\beta\gamma$ purified from rat brain (Katada *et al.*, 1987) is not known.

6. PT-substrate G-proteins are also coupled to ion channels in a positive or negative manner without mediation of cAMP or other intracellular messengers. Muscarinic (M_2) receptor-linked activation of potassium channels is blocked by PT in rat atrial cells. Likewise, calcium channels are coupled to a GTP-binding protein in certain endocrine cells in a manner susceptible to PT treatment of cells (Ui, 1986). These PT-substrate G-proteins are not yet identified, and hence are often referred to as G_x . A G-protein in sea urchin eggs is also capable of being ADP-ribosylated by PT (Oinuma *et al.*, 1986).
7. G-proteins undergo reversible transition between the trimer and dimer, i.e. between GDP-bound $\alpha\beta\gamma$ and GTP-bound α and $\beta\gamma$, which is related to the mechanism for these G-proteins to act as the transducer (Katada *et al.*, 1986a). The GDP-bound $\alpha\beta\gamma$ is associated with a receptor, thereby increasing the affinity of the receptor for its specific agonist. This GDP-bound trimer form is an inactive state of the G-protein in the sense that it is not interacting with effector. Stimulation of the receptor by the agonist results in its dissociation from the G-protein and the displacement of GDP by cytosolic GTP on α which is in turn resolved from $\beta\gamma$. This is the active state of the G-protein, since either GTP-bound α or $\beta\gamma$ thus liberated from the receptor is capable of direct interaction with effector. GTP is then hydrolysed to GDP on α due to its GTP-hydrolysing activity; the resultant GDP-bound α is quickly re-associated with $\beta\gamma$ to recover the initial inactive state of GDP-bound trimer. Thus, signals are transduced from receptor to effector by repetition of this cycle between GDP-bound $\alpha\beta\gamma$ and GTP-bound α and $\beta\gamma$.
8. Finally, only a bare mention will be made below of G-proteins not serving as the substrate of PT. The protein encoded by a *ras* gene, one of the oncogenes responsible for mammalian cell proliferation, is also a GTP-binding protein, which is referred to as p21 based on its M_r value. Just like the α -subunits of PT-substrate G-proteins, there is a cysteine residue at the

000, despite the fact that cAMP is (Okajima and Ui, 1984; Nakamura). Later studies revealed that the PT lipase C directly in these cell types (1985; Kikuchi *et al.*, 1986). Likewise, pressed by PT when DNA synthesis factors (Murayama and Ui, 1987b). is far for or against the idea that the of signalling is identical with G_i, G_s above. Recently, a novel G-protein, cells that had been differentiated to phoxide (Oinuma *et al.*, 1987) but its r brain (Katada *et al.*, 1987) is not

led to ion channels in a positive or cAMP or other intracellular messenger activation of potassium channels is wise, calcium channels are coupled toocrine cells in a manner susceptible to PT-substrate G-proteins are not yet l to as G_x . A G-protein in sea urchin ylated by PT (Oinuma *et al.*, 1986). n between the trimer and dimer, i.e. ound α and $\beta\gamma$, which is related to act as the transducer (Katada *et al.*, sociated with a receptor, thereby for its specific agonist. This GDP- of the G-protein in the sense that it ation of the receptor by the agonist rotein and the displacement of GDP i resolved from $\beta\gamma$. This is the active P-bound α or $\beta\gamma$ thus liberated from raction with effector. GTP is then P-hydrolysing activity; the resultant with $\beta\gamma$ to recover the initial inactive als are transduced from receptor to erween GDP-bound $\alpha\beta\gamma$ and GTP-

made below of G-proteins not serving
encoded by a *ras* gene, one of the
cell proliferation, is also a GTP-
p21 based on its M_r value. Just like
ins, there is a cysteine residue at the

fourth position from the carboxyl terminus of p21. This cysteine is, however, not ADP-ribosylated by PT, but instead is acylated by a fatty acid which plays an important rôle in attachment of p21 to the inner surface of plasma membranes of mammalian cells. In the case of the yeast, *Saccharomyces cerevisiae*, the *ras* gene codes for a GTP-binding protein which is larger in molecular size than p21 and acts as a regulator of adenylate cyclase. This G-protein in yeast is again not ADP-ribosylated by PT or other bacterial toxins.

8. SUMMARY

Most of the recent studies on PT have been accomplished with preparations which were purified on the basis of its activity as islet-activating protein (IAP). PT thus purified possesses a complicated subunit structure that is responsible for the occurrence of diverse biological activities of the toxin such as those of LPF, HSF, HA, MPA, adjuvant, mitogen, and IAP.

PT is a hexamer of five dissimilar subunits: S1, S2, S3, S4, and S5. It is one of the A-B toxins (Gill, 1978). The biggest subunit, S1, is referred to as the A-protomer, while the residual five subunits constitute the B-oligomer in such a manner that two dimers, D1 composed of S2 and S4 and D2 composed of S3 and S4, are connected with each other by means of S5, the smallest subunit. The B-oligomer binds via the two dimers to particular glycoproteins on the surface of a variety of mammalian cells. This binding is the first step of the development of biological activities of the toxin in mammals. The free ϵ -amino groups of the lysine residues in peptides play an essential rôle in the attachment of D2 to the cell surface but not in the attachment of D1. The blockade of these amino groups by chemical modification revealed the dual mechanisms of B-oligomer binding to target cells and the relative rôles of the two dimers therein.

One of the dual binding mechanisms is responsible for the ADP-ribosylation of cellular proteins. In this case, the B-oligomer binds to a site on the cell surface via D1 or D2, although the affinity of D1 for this one-point attachment is higher than D2. This type of binding results in internalization of the A-protomer into the target cells. The slow internalization is reflected in the lag time preceding the onset of biological activities related to ADP-ribosylation *in vivo* or in intact cells *in vitro*. The A-protomer, after entry, undergoes intracellular processing to be converted to the active ADP-ribosyltransferase. This processing can be mimicked by the incubation of the naive PT with ATP and dithiothreitol. The ADP-ribosylation of particular membrane proteins, GTP-binding proteins (G-proteins), by this enzyme is the mechanism for the activities of PT listed under 'Class 1' and 'Class 3' in Table 1.

ADP-ribosylation proceeds selectively at the cysteine residue of the α -subunit of the toxin-substrate G-proteins. In a variety of mammalian cells, the G-

proteins transduce extracellular signals to the intracellular effector systems under physiological conditions, but are not able to play this rôle any longer after being ADP-ribosylated. The thus-elicted blockade of cellular signalling at the membrane transducer level is responsible for development of 'Class 1' and 'Class 3' activities of PT.

Another binding mechanism is the divalent or two-point attachment of the B-oligomer via D1 and D2 to the cell surface; D2 shows a higher affinity than D1 for their own binding sites. This divalent binding produces cross-linking of glycoproteins on the cell surface, which triggers, by itself, a cascade of intracellular signalling processes eventually leading to proliferation of lymphocytes, glucose utilization by adipocytes and contraction of endothelial cells, etc. This is the mechanism underlying the 'Class 2' activities of PT in Table 1. Since free amino groups of the lysine residues in the D2 molecule are essential for its binding to the cells, none of the 'Class 2' activities were provoked by dimerhylated or acetamidinated PT.

ADP-ribosylation catalysed by the A-protomer is not involved in the 'Class 2' activities. The actions *in vitro* of PT listed under the category of 'Class 2' are, therefore, mimicked by the B-oligomer separated from the holotoxin. The B-oligomer is, however, unstable *in vivo* unless it is associated with the A-protomer and alone is not capable of producing 'Class 2' activities *in vivo*.

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REFERENCES

- Asano, T., Ui, M., and Ogawara, N. (1985). Prevention of the agonist binding to γ -aminobutyric acid B receptors by guanine nucleotides and islet-activating protein, pertussis toxin, in bovine cerebral cortex. Possible coupling of the toxin-sensitive GTP-binding proteins to receptors. *J. Biol. Chem.*, 260, 12 653-12 658.
- Bokoch, G. M., Katada, T., Northup, J. K., Ui, M., and Gilman, A. G. (1984). Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J. Biol. Chem.*, 259, 3560-3567.
- Gill, D. M. (1978). Seven toxin peptides that cross cell membranes. In *Bacterial Toxins and Cell Membranes* (Eds J. Jellaszewicz and T. Wadstrom), pp. 291-331. Academic Press, New York.
- Gilman, A. G. (1984). G proteins and dual control of adenylate cyclase. *Cell*, 36, 577-579.
- Haga, K., Haga, T., Ichiyama, A., Katada, T., Kurose, H., and Ui, M. (1985).

c intracellular effector systems ble to play this rôle any longer l blockade of cellular signalling le for development of 'Class 1'

or two-point attachment of the D2 shows a higher affinity than binding produces cross-linking riggers, by itself, a cascade of ding to proliferation of lympho- traction of endothelial cells, etc. 2' activities of PT in Table 1. in the D2 molecule are essential : 2' activities were provoked by

ner is not involved in the 'Class under the category of 'Class 2' parated from the holotoxin. The ess it is associated with the A-g 'Class 2' activities *in vivo*.

MENTS

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S

vention of the agonist binding to γ - cotides and islet-activating protein, ible coupling of the toxin-sensitive n., 260, 12 653-12 658.

i, M., and Gilman, A. G. (1984). anine nucleotide-binding regulatory 259, 3560-3567.

cell membranes. In *Bacterial Toxins* (Wadstrom), pp. 291-331. Academic

rol of adenylate cyclase. *Cell*, 36,

, Kurose, H., and Ui, M. (1985).

Functional reconstitution of purified muscarinic receptors and inhibitory guanine nucleotide regulatory protein. *Nature*, 316, 731-732.

Hazeki, O., and Ui, M. (1981). Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. *J. Biol. Chem.*, 256, 2856-2862.

Irons, L. I., and MacLennan, A. P. (1979). Isolation of the lymphocytosis promoting factor-haemagglutinin of *Bordetella pertussis* by affinity chromatography. *Biochim. Biophys. Acta*, 580, 175-185.

Itoh, H., Okajima, F., and Ui, M. (1984). Conversion of adrenergic mechanism from an α - to a β -type during primary culture of rat hepatocytes. *J. Biol. Chem.*, 259, 15 464-15 473.

Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K., and Kaciro, Y. (1986). Molecular cloning and sequence determination of cDNAs coding for α -subunits of G_i , G_o , and G_q proteins from rat brain. *Proc. Natl. Acad. Sci. USA*, 83, 3776-3780.

Katada, T., and Ui, M. (1976). Accelerated turnover of blood glucose in pertussis-sensitized rats due to combined actions of endogenous insulin and adrenergic beta-stimulation. *Biochim. Biophys. Acta*, 421, 57-69.

Katada, T., and Ui, M. (1977). Perfusion of the pancreas isolated from pertussis-sensitized rats: potentiation of insulin secretory responses due to β -adrenergic stimulation. *Endocrinology*, 101, 1247-1255.

Katada, T., and Ui, M. (1979a). Islet-activating protein. Enhanced insulin secretion and cAMP accumulation in pancreatic islets due to activation of native Ca ionophores. *J. Biol. Chem.*, 254, 469-479.

Katada, T., and Ui, M. (1979b). Effect of *in vivo* pretreatment of rats with a new protein purified from *Bordetella pertussis* on *in vitro* secretion of insulin: role of calcium. *Endocrinology*, 104, 1822-1827.

Katada, T., and Ui, M. (1980). Slow interaction of islet-activating protein with pancreatic islets during primary culture to cause reversal of α -adrenergic inhibition of insulin secretion. *J. Biol. Chem.*, 255, 9580-9588.

Katada, T., and Ui, M. (1981a). Islet-activating protein. A modifier of receptor-mediated regulation of rat islet adenylate cyclase. *J. Biol. Chem.*, 256, 8310-8317.

Katada, T., and Ui, M. (1981b). *In vitro* effects of islet-activating protein on cultured rat pancreatic islets. Enhancement of insulin secretion, adenosine 3':5'-monophosphate accumulation and ^{45}Ca flux. *J. Biochem.*, 89, 979-990.

Katada, T., and Ui, M. (1982a). Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. USA*, 79, 3129-3133.

Katada, T., and Ui, M. (1982b). ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J. Biol. Chem.*, 257, 7210-7216.

Katada, T., Amano, T., and Ui, M. (1982). Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. *J. Biol. Chem.*, 257, 3739-3746.

Katada, T., Tamura, M., and Ui, M. (1983). The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch. Biochem. Biophys.*, 224, 290-298.

Katada, T., Bokoch, G. M., Northup, J. K., Ui, M., and Gilman, A. G. (1984a). The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Properties and function of the purified protein. *J. Biol. Chem.*, 259, 3568-3577.

Katada, T., Bokoch, G. M., Smigel, M. D., Ui, M., and Gilman, A. G. (1984b). The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase.

- Subunit dissociation and the inhibition of adenylate cyclase in S49 lymphoma cytochrome c and wild type membranes. *J. Biol. Chem.*, 259, 3586-3595.
- Katada, T., Northup, J. K., Bokoch, G. M., Ui, M., and Gilman, A. G. (1984c). The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J. Biol. Chem.*, 259, 3578-3585.
- Katada, T., Oinuma, M., and Ui, M. (1986a). Mechanisms for inhibition of the catalytic activity of adenylate cyclase by the guanine nucleotide-binding proteins serving as the substrate of islet-activating protein, pertussis toxin. *J. Biol. Chem.*, 261, 5215-5221.
- Katada, T., Oinuma, M., and Ui, M. (1986b). Two guanine nucleotide-binding proteins in rat brain serving as the specific substrate of islet-activating protein, pertussis toxin. Interaction of the α -subunits with β -subunits in development of their biological activities. *J. Biol. Chem.*, 261, 8182-8191.
- Katada, T., Oinuma, M., Kusakabe, K., and Ui, M. (1987). A new GTP-binding protein in brain tissues serving as the specific substrate of islet-activating protein, pertussis toxin. *FEBS Lett.*, 213, 353-358.
- Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M., and Takai, Y. (1986). Direct evidence for involvement of guanine nucleotide-binding protein in chemotactic peptide-stimulated formation of inositol bisphosphate and trisphosphate in differentiated human leukemia (F.L-60) cells. Reconstitution with G_i or G_o of the plasma membranes ADP-ribosylated by pertussis toxin. *J. Biol. Chem.*, 261, 11558-11562.
- Kurose, H., Katada, T., Amano, T., and Ui, M. (1983). Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α -adrenergic, cholinergic, and opiate receptors in neuroblastoma x glioma hybrid cells. *J. Biol. Chem.*, 258, 4870-4875.
- Kurose, H., Katada, T., Haga, T., Haga, K., Ichiyama, A., and Ui, M. (1986). Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J. Biol. Chem.*, 261, 6423-6428.
- Kurose, H., and Ui, M. (1983). Functional uncoupling of muscarinic receptors from adenylate cyclase in rat cardiac membranes by the active component of islet-activating protein, pertussis toxin. *J. Cyc. Nuc. Prot. Phos. Res.*, 91, 305-318.
- Kurose, H., and Ui, M. (1985). Dual pathways of receptor-mediated cyclic GMP generation in NG108-15 cells as differentiated by susceptibility to islet-activating protein, pertussis toxin. *Arch. Biochem. Biophys.*, 238, 424-434.
- Locht, C., and Keith, J. M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science*, 232, 1258-1264.
- Murayama, T., and Ui, M. (1983). Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP-ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *J. Biol. Chem.*, 258, 3319-3326.
- Murayama, T., and Ui, M. (1983). [³H]GDP release from rat and hamster adipocyte membranes independently linked to receptors involved in activation or inhibition of adenylate cyclase. *J. Biol. Chem.*, 259, 761-769.
- Murayama, T., and Ui, M. (1985). Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. Selective susceptibility to islet-activating protein, pertussis toxin. *J. Biol. Chem.*, 260, 7226-7233.
- Murayama, T., and Ui, M. (1987a). Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. *J. Biol. Chem.*, 262, 5522-5529.
- Murayama, T., and Ui, M. (1987b). Possible involvement of a GTP-binding protein,

Multiple Biological Activities of Pertussis Toxin

143

- late cyclase in S49 lymphoma cyc-3586-3595.
- 4, and Gilman, A. G. (1984c). The γ component of adenylate cyclase: resistant hormonal inhibition. *J. Biol. Chem.*, 259, 5215-5221.
- mechanisms for inhibition of the catalytic subunit of adenylate cyclase by the γ component of pertussis toxin. *J. Biol. Chem.*, 259, 5215-5221.
- guanine nucleotide-binding proteins: islet-activating protein, pertussis toxin, and their involvement in development of their biological activities. *J. Biol. Chem.*, 259, 5215-5221.
- Ueda, M. (1987). A new GTP-binding protein: islet-activating protein, pertussis toxin. *J. Biol. Chem.*, 262, 3584-3593.
- Ueda, M., and Takai, Y. (1986). Islet-activating protein in chemotactic peptide and trisphosphate in differentiation with G_i or G_o of the plasma membrane. *J. Biol. Chem.*, 261, 11 558-11 562.
- (1983). Specific uncoupling by islet-activating protein of α -adrenergic, muscarinic, and glioma hybrid cells. *J. Biol. Chem.*, 258, 11 558-11 562.
- Ichijima, A., and Ui, M. (1986). Islet-activating protein with purified inhibitory function in phospholipid vesicles. *J. Biol. Chem.*, 261, 11 558-11 562.
- Coupling of muscarinic receptors from guinea pig brain to islet-activating protein. *Regul. Pept.*, 91, 305-318.
- Effect of receptor-mediated cyclic GMP production by susceptibility to islet-activating protein. *J. Biol. Chem.*, 259, 424-434.
- Islet-activating protein gene: nucleotide sequence and expression. *J. Biol. Chem.*, 260, 7226-7233.
- Inhibitory function of the guanine nucleotide-binding protein due to its ADP-ribosylation by pertussis toxin. *J. Biol. Chem.*, 258, 11 558-11 562.
- Release from rat and hamster adipocyte membranes of islet-activating protein involved in activation or inhibition of adenylate cyclase. *J. Biol. Chem.*, 259, 424-434.
- Regulated inhibition of adenylate cyclase in 3T3 fibroblasts. Selective susceptibility to pertussis toxin. *J. Biol. Chem.*, 260, 7226-7233.
- Arachidonic acid may stimulate membrane receptor-mediated phospholipid breakdown in 3T3 fibroblasts. *J. Biol. Chem.*, 259, 424-434.
- Involvement of a GTP-binding protein, pertussis toxin, in receptor-mediated signalling responsible for cell proliferation. *J. Biol. Chem.*, 262, 12463-12467.
- Murayama, T., Katada, T., and Ui, M. (1983). Guanine nucleotide activation and inhibition of adenylate cyclase as modified by islet-activating protein, pertussis toxin, in mouse 3T3 fibroblasts. *Arch. Biochem. Biophys.*, 221, 381-390.
- Nakamura, T., and Ui, M. (1983). Suppression of passive cutaneous anaphylaxis by pertussis toxin, an islet-activating protein, as a result of inhibition of histamine release from mast cells. *Biochem. Pharmacol.*, 32, 3435-3441.
- Nakamura, T., and Ui, M. (1984). Islet-activating protein, pertussis toxin, inhibits Ca^{2+} -induced and guanine nucleotide-dependent releases of histamine and arachidonic acid from rat mast cells. *FEBS Lett.*, 173, 414-418.
- Nakamura, T., and Ui, M. (1985). Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release, and histamine secretion in mast cells by islet-activating protein, pertussis toxin. A possible involvement of the toxin-specific substrate in the Ca^{2+} -mobilizing receptor-mediated biosignaling system. *J. Biol. Chem.*, 260, 3584-3593.
- Nicosia, A., Perugini, M., Franzini, C., Casagli, M. C., Borri, M. G., Antoni, G., Almon, M., Neri, P., Ratti, G., and Rappuoli, R. (1986). Cloning and sequencing of the pertussis toxin gene: operon structure and gene duplication. *Proc. Natl. Acad. Sci. USA*, 83, 4631-4635.
- Nogimori, K., Ito, K., Tamura, M., Satoh, S., Ishii, S., and Ui, M. (1984a). Chemical modification of islet-activating protein, pertussis toxin. Essential role of free amino groups in its lymphocytosis-promoting activity. *Biochim. Biophys. Acta*, 801, 220-231.
- Nogimori, K., Tamura, M., Yajima, M., Ito, K., Nakamura, T., Kajikawa, N., Maruyama, Y., and Ui, M. (1984b). Dual mechanisms involved in development of diverse biological activities of islet-activating protein, pertussis toxin, as revealed by chemical modification of lysine residues in the toxin molecule. *Biochim. Biophys. Acta*, 801, 232-243.
- Nogimori, K., Tamura, M., Nakamura, T., Yajima, M., Ito, K., and Ui, M. (1986a). Dual mechanisms involved in the development of diverse biological activities of islet-activating protein, pertussis toxin, as revealed by chemical modification of the toxin molecule. *Develop. Biol. Standard.*, 61, 51-61.
- Nogimori, K., Tamura, M., Yajima, M., Hashimura, N., Ishii, S., and Ui, M. (1986b). Structure-function relationship of islet-activating protein, pertussis toxin: biological activities of hybrid toxins reconstituted from native and methylated subunits. *Biochemistry*, 25, 1355-1363.
- Ohta, H., Okajima, F., and Ui, M. (1985). Inhibition by islet-activating protein of a chemotactic peptide-induced early breakdown of inositol phospholipids and Ca^{2+} mobilization in guinea pig neutrophils. *J. Biol. Chem.*, 260, 15 771-15 780.
- Oinuma, M., Katada, T., Yokosawa, H., and Ui, M. (1986). Guanine nucleotide-binding protein in sea urchin eggs serving as the specific substrate of islet-activating protein, pertussis toxin. *FEBS Lett.*, 207, 28-34.
- Oinuma, M., Katada, T., and Ui, M. (1987). A new GTP-binding protein in differentiated human leukemic (HL-60) cells serving as the specific substrate of islet-activating protein, pertussis toxin. *J. Biol. Chem.*, 262, 8347-8353.
- Okajima, F., and Ui, M. (1984). ADP-ribosylation of the specific membrane protein by islet-activating protein, pertussis toxin, associated with inhibition of a chemotactic peptide-induced arachidonate release in neutrophils. A possible role of the toxin substrate in Ca^{2+} -mobilizing biosignaling. *J. Biol. Chem.*, 259, 13 863-13 871.
- Okajima, F., Katada, T., and Ui, M. (1985). Coupling of the guanine nucleotide regulatory protein to chemotactic peptide receptors in neutrophil membranes and

- uncoupling by islet-activating protein, pertussis toxin. A possible role of the toxin substrate in Ca^{2+} -mobilizing receptor-mediated signal transduction. *J. Biol. Chem.*, 260, 6761-6768.
- Sugimoto, M., Nakanishi, Y., Orokawa, M., Uchida, N., Yasuda, T., Sato, H., and Sato, Y. (1983). Effect of *Bordetella pertussis* leukocytosis (lymphocytosis)-promoting factor on the physical lymphoepithelial-cell association studied with the use of an *in vitro* model of mouse thymus. *J. Immunol.*, 130, 2767-2774.
- Sumi, T., and Ui, M. (1975). Potentiation of the adrenergic β -receptor-mediated insulin secretion in pertussis-sensitized rats. *Endocrinology*, 97, 352-358.
- Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M., and Ishii, S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry*, 21, 5516-5522.
- Tamura, M., Nogimori, K., Yajima, M., Ase, K., and Ui, M. (1983). A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. *J. Biol. Chem.*, 258, 6756-6761.
- Tsuda, M., Tsuda, T., Terayama, Y., Fukada, Y., Akino, T., Yamanaka, G., Stryer, L., Katada, T., Ui, M., and Ebrey, T. (1986). Kinship of cephalopod photoreceptor G-protein with vertebrate transducin. *FEBS Lett.*, 198, 5-10.
- Ui, M. (1984). Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. *Trends Pharmacol. Sci.*, 5, 277-279.
- Ui, M. (1986). Pertussis toxin as a probe of receptor coupling to inositol lipid metabolism. In *Receptor Biochemistry and Methodology*, Vol. 7 (Ed. J. W. Putney), *Phosphoinositides and Receptor Mechanisms*, pp. 163-195. Alan R. Liss, Inc., New York.
- Ui, M., Katada, T., Murayama, T., and Kurose, H. (1984a). Selective blockage by islet-activating protein, pertussis toxin, of negative signal transduction from receptors to adenylate cyclase. In *Neurotransmitter Receptors* (Eds S. Kito, T., Segawa, K., Kuriyama, H. I. Yamamura, and R. W. Olsen), pp. 1-16, Plenum Publishing Corp., New York.
- Ui, M., Katada, T., Murayama, T., and Kurose, H. (1984b). Chemical modification of nucleotide regulatory proteins catalyzed by bacterial toxins. In *Proceedings of IUPHAR 9th International Congress of Pharmacology*, vol. 1 (Eds W. Paton, J. Mitchell, and P. Turner), pp. 271-278. Macmillan Press, London.
- Ui, M., Katada, T., Murayama, T., and Kurose, H. (1984c). Role of N protein in coupling of adrenergic receptors and adenylate cyclase. In *Endocrinology* (Eds F. Labrie and L. Proulx), pp. 157-160. Excerpta Medica, Amsterdam.
- Ui, M., Katada, T., Murayama, T., Kurose, H., Yajima, M., Tamura, M., Nakamura, T., and Nogimori, K. (1984d). Islet-activating protein, pertussis toxin: a specific uncoupler of receptor-mediated inhibition of adenylate cyclase. In *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, vol. 17 (Eds P. Greengard, G. A. Robison, R. Paoletti, and S. Nicosia), pp. 145-151. Raven Press, New York.
- Ui, M., Katada, T., Murayama, T., and Nakamura, T. (1984e). Islet-activating protein, pertussis toxin, as a probe for receptor-mediated signal transduction. In *Calcium Regulation in Biological Systems* (Eds S. Ebashi, M. Endo, K. Imahori, S. Kakiuchi, and Y. Nishizuka), pp. 157-169. Academic Press, New York.
- Ui, M., Katada, T., and Tamura, M. (1985a). ADP-ribosylation of a membrane protein catalyzed by islet-activating protein, pertussis toxin. In *ADP-Ribosylation of Proteins* (Eds F. R. Althaus, H. Hiltz, and S. Shalf), pp. 551-561. Springer-Verlag, Berlin.
- Ui, M., Nogimori, K., and Tamura, M. (1985b). Islet-activating protein, pertussis toxin: subunit structure and mechanism for its multiple biological actions. In *Pertussis*

- hida, N., Yasuda, T., Sato, H., and Ikocytosis (lymphocytosis)-promoting action studied with the use of an in 1, 2767-2774.
- adrenergic B-receptor-mediated insulin 97, 352-358.
- U., Ito, K., Katada, T., Ui, M., and cleaving protein, pertussis toxin, in 11, 5516-5522.
- , and Ui, M. (1983). A role of the B-russis toxin, in development of the 258, 6756-6761.
- , Akino, T., Yamanaka, G., Stryer, Kinship of cephalopod photoreceptor 11, 198, 5-10.
- toxin: a probe for functions of the ent of adenylate cyclase. *Trends Phar-*
- ptor coupling to inositol lipid metab-, Vol. 7 (Ed. J. W. Putney), *Phospho-* 11, Alan R. Liss, Inc., New York.
- e, H. (1984a). Selective blockage by ive signal transduction from receptors 11, (Eds S. Kito, T., Segawa, K., , pp. 1-16, Plenum Publishing Corp.,
- e, H. (1984b). Chemical modification y bacterial toxins. In *Proceedings of macology*, vol. 1 (Eds W. Paton, J. illan Press, London.
- se, H. (1984c). Role of N protein in te cyclase. In *Endocrinology* (Eds F. Medica, Amsterdam.
- Yajima, M., Tamura, M., Nakamura, ig protein, pertussis toxin: a specific denylate cyclase. In *Advances in Cyclic h*, vol. 17 (Eds P. Greengard, G. A. -151. Raven Press, New York.
- ra, T. (1984e). Islet-activating protein, ated signal transduction. In *Calcium* , M. Endo, K. Imahori, S. Kakiuchi, ress, New York.
- DP-ribosylation of a membrane protein oxin. In *ADP-Ribosylation of Proteins* p. 551-561. Springer-Verlag, Berlin.
- 5b). Islet-activating protein, pertussis multiple biological actions. In *Pertussis*
- Toxin (Eds R. D. Sekwa, J. Moss, and M. Vaughan), pp. 19-43. Academic Press, New York.
- Ui, M., Okajima, F., and Itoh, H. (1985c). ADP-ribosylation of the inhibitory guanine nucleotide regulatory protein (N_i) as a possible mechanism underlying development of β -adrenergic responses during primary culture of rat hepatocytes. In *Advances in Cyclic Nucleotides and Protein Phosphorylation Research*, vol. 19 (Eds D. M. F. Cooper and K. B. Seamon), pp. 195-205. Raven Press, New York.
- Ui, M., Okajima, F., Murayama, T., Nakamura, T., Kurose, H., Itoh, H., and Ohta, H. (1985d). A role of the inhibitory guanine nucleotide-binding regulatory protein in signal transduction via Ca^{2+} -mobilizing receptors. In *Adrenergic Receptors: Molecular properties and therapeutic implications* (Eds R. J. Lefkowitz, and E. Lindénlaub), pp. 209-258. F. K. Schattauer Verlag, Stuttgart and New York.
- Ui, M., Katada, T., and Kurose, H. (1986a). Islet-activating protein, pertussis toxin, as a probe for the biosignaling system. In *Natural Products and Biological Activities* (Eds H. Imura, T. Goto, T. Murachi, and T. Nakajima), pp. 247-258. University of Tokyo Press, Tokyo.
- Ui, M., Nogimori, K., and Tamura, M. (1986b). Structure of islet-activating protein, pertussis toxin, required for its multiple biological activities. In *Bacterial Protein Toxins* (Eds P. Palmagne, J. E. Alouf, F. J. Fehrenbach, J. J. Jelszewicz, and M. Thelestam), pp. 9-17. Gustav Fischer Verlag, Stuttgart and New York.
- Yajima, M., Hosoda, K., Kanbayashi, Y., Nakamura, T., Nogimori, K., Nakase, Y., and Ui, M. (1978a). Islet-activating protein in *Bordetella pertussis* that potentiates insulin secretory responses of rat. Purification and characterization. *J. Biochem.*, 83, 295-303.
- Yajima, M., Hosoda, K., Kanbayashi, Y., Nakamura, T., Takahashi, I., and Ui, M. (1978b). Biological properties of islet-activating protein purified from the culture medium of *Bordetella pertussis*. *J. Biochem.*, 83, 305-312.
- Yajima, M., Kurokawa, S., and Takahashi, I. (1981). Involvement of endogenous epinephrine in histamine-induced hyperinsulinemia and hypoglycemia in rats treated with islet-activating protein. *Biochem. Pharmacol.*, 30, 3305-3309.
- Yamazaki, S., Katada, T., and Ui, M. (1982). α_2 -Adrenergic inhibition of insulin secretion via interference with cAMP generation in rat pancreatic islets. *Mol. Pharmacol.*, 21, 648-653.

the receptor under non-denaturing conditions is estimated to be 1000 kD, about four times the apparent mass of the dissociated chains under denaturing conditions.

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ADP-Ribosyltransferase Activity of Pertussis Toxin and Immunomodulation by *Bordetella pertussis*

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Pertussis toxin is produced by the causative agent of whooping cough, *Bordetella pertussis*, and is an adenosine diphosphate (ADP)-ribosyltransferase capable of covalently modifying and thereby inactivating many eukaryotic G proteins involved in cellular metabolism. The toxin is a principal determinant of virulence in whooping cough and is a primary candidate for an acellular pertussis vaccine, yet it is unclear whether the ADP-ribosyltransferase activity is required for both pathogenic and immunoprotective activities. A *B. pertussis* strain that produced an assembled pertussis holotoxin with only 1 percent of the ADP-ribosyltransferase activity of the native toxin was constructed and was found to be deficient in pathogenic activities associated with *B. pertussis* including induction of leukocytosis, potentiation of anaphylaxis, and stimulation of histamine sensitivity. Moreover, this mutant strain failed to function as an adjuvant and was less effective in protecting mice from intracerebral challenge infection. These data suggest that the ADP-ribosyltransferase activity is necessary for both pathogenicity and optimum immunoprotection. These findings bear directly on the design of a nontoxic pertussis vaccine.

PERTUSSIS TOXIN IS THE PRIMARY determinant of virulence produced by *Bordetella pertussis* in whooping cough (2-3). Aspects of the systemic pathology of the disease, including lymphocytosis and hypoglycemia, can be reproduced in laboratory animals with purified toxin alone (4). The toxin is composed of five dissimilar polypeptides that can be divided into two functional subunits (5); an "A" monomer, S1, mediates adenosine diphosphate (ADP)-ribosylation of host G proteins (6), and a "B" oligomer, composed of four different polypeptides, designated S2 through S5, mediates binding of the toxin to host tissue (7). Two molecular mechanisms of pathogenesis have been proposed for pertus-

sis toxin. The first is the ADP-ribosylation and concomitant inactivation of host G proteins involved in normal eukaryotic cell metabolism (6). The second mechanism is the lectin-like binding of the B oligomer to eukaryotic cells (7), which has been proposed to act mitogenically to cause the lymphocytosis and other immunomodulatory activities mediated by pertussis toxin (8).

Pertussis toxin is also found in, and is considered to be a primary protective component of, both the traditional whole-cell (2, 9) and the newer acellular (10) formulations of the pertussis vaccine. However, there is speculation that active toxin present in the vaccines may cause certain rare but serious vaccination sequelae including hypotonic, hyporesponsive syndrome, convulsions, and encephalopathy (11). Recent efforts to clone the toxin genes (12) are in part predicated on the proposition that an enzymatically inactive version of the toxin molecule produced by modified toxin genes might serve as a valuable component in a defined vaccine. We were interested in determining the contribution of the ADP-ribosyltransferase activity to pathogenesis and immunoprotection and so constructed *B. pertussis* strains with defined mutations in the toxin genes. These genes were assayed

for the induction of leukocytosis (4, 13), the potentiation of anaphylaxis (4, 14, 15), and the stimulation of histamine sensitivity (4). We also examined the capacity of the strains to serve as adjuvants (4) and their immunoprotective activity against experimental *B. pertussis* infection in mice (16).

A *B. pertussis* strain with a nonpolar mutation that altered the primary structure of the pertussis toxin S1 or ADP-ribosyltransferase subunit was constructed by in vitro linker scanning mutagenesis (17), followed by allelic exchange (18, 19) of the mutation into the *B. pertussis* chromosome. This mutation, *ptx*A3201, introduced a 12-bp insertion at the Sal I restriction site of the S1 gene (Fig. 1), maintaining the reading frame integrity and introducing four novel codons, for Val-Asp-Gly-Ser, between Tyr¹⁴¹ and Val¹⁴² (12). We chose this site for modification because of its proximity to Glu¹⁴⁰; Collier and co-workers have shown that for each of two other ADP-ribosyltransferase toxins, diphtheria toxin and pseudomonas exopro-

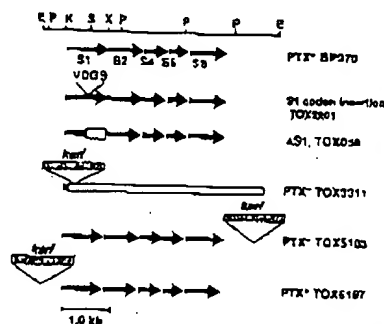


Fig. 1. Pertussis toxin operon mutations. Defined mutations in the pertussis toxin operon were constructed in vitro by means of standard recombinant DNA technology (30) and introduced into the chromosome of *B. pertussis* strain BP370 (18) by allelic exchange (18, 19). The parental *B. pertussis* strain, PTX⁺, BP370, contains a polycistronic arrangement of the genes for the five toxin polypeptide subunits (12, 18). The S1 codon insertion derivative, TOX3201, contains a 12-bp insertion, GACGGATCCGTC, at the Sal I site in the S1 gene, introducing the amino acids Val-Asp-Gly-Ser into the S1 polypeptide between Tyr¹⁴¹ and Val¹⁴² (12). The Δ S1 derivative, TOX058, contains a deletion of the 3' half of the S1 gene, from the Sal I site to the Xba I site, fusing the S1 codon for Asp¹⁴³ to the stop codon in the Xba I site. The construction of TOX3201 (19) and TOX058 (27) is described in greater detail elsewhere. TOX3201 was previously designated BP370 Δ ptx-3201 (19). The PTX⁻ derivative, TOX3211, has a deletion extending from about 200 bp inside the 5' end of the S1 gene down through about 1100 bp 3' of the S3 gene, and a *kan*^r gene (26) ligated into the breach (18). The PTX⁻, TOX5105 derivative has an insertion of the *kan*^r gene about 800 bp 3' of the toxin structural genes (18). The PTX⁺, TOX5167 derivative has an insertion of the *kan*^r gene about 400 bp 5' of the toxin structural genes (18).

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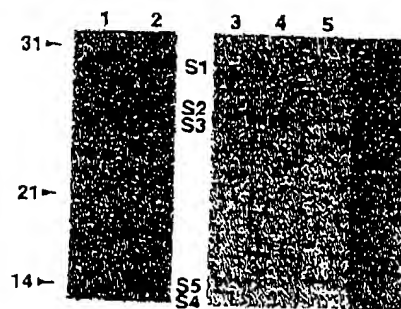


Fig. 2. TOX3201 export and assembly. Pertussis toxins were purified from culture supernatants of strains BP370 and TOX3201 by ferritin-Sepharose affinity chromatography (22). On analysis by SDS-polyacrylamide gel electrophoresis (23) and Coomassie blue staining, both the TOX3201 (lane 1) and the BP370 (lane 2) toxins exhibited all of the subunits of a complete holotoxin structure (5, 22). Western immunoblot (24) analysis of the TOX3201 toxin showed reactivity with monoclonal antibodies to the S1 (lane 3), S2 (lane 4), and S4 (lane 5) subunits of pertussis toxin. Molecular mass standards are indicated by arrows and are given in kilodaltons.

tein A, a Glu residue is a critical component of the enzymatic active sites (20). Chou and Fasman analysis (21) of the Glu¹⁴⁰-Tyr¹⁴¹-Val¹⁴² region of the S1 subunit of pertussis toxin predicted beta structure that the four-amino acid insertion of *pxcA3201* would interrupt with a turn.

The toxin molecules produced by the codon insertion mutant strain, TOX3201, and the parental strain, BP370 (18), were purified from culture supernatant by ferritin-Sepharose affinity chromatography (22) for comparison by SDS-polyacrylamide gel electrophoresis (23) and Western immunoblot (24) (Fig. 2). The toxin molecule produced by TOX3201, which we designated CRM3201, has an S1 subunit of a larger apparent molecular weight than the native toxin S1 subunit. This appropriately reflected the insertion of four amino acids into the S1 polypeptide of CRM3201. The CRM3201 molecule was also found to contain the polypeptides of the toxin B oligomer, S2 through S5, and was found to be equivalent to the native toxin in its ability to hemagglutinate goose erythrocytes (22). CRM3201 had, however, only 1% of the ADP-ribosyltransferase activity of the native toxin as assayed by the ADP-ribosylation of transducin (25). The *pxcA3201* mutation thus may define a region of the S1 polypeptide involved in this enzymatic activity. In sum, these data suggest that the CRM3201 toxin molecule is exported as an assembled holotoxin with a functional B oligomer but a substantially less active S1 ADP-ribosyltransferase subunit.

In assays for biological activities, several

other *B. pertussis* strains were selected for comparison with TOX3201 (Fig. 1). These included a nontoxinogenic strain, TOX3311 (18), containing a kanamycin resistance (*kan^r*) gene (26) inserted in place of the toxin operon, and two toxinogenic *B. pertussis* strains, TOX5105 and TOX5167 (18), containing insertions of the *kan^r* gene outside of the toxin operon. We also tested strain TOX058, in which the 3' half of the S1 gene, from the Sal I to the Xba I restriction sites, had been deleted. The construction and characterization of TOX058 will be described in detail elsewhere (27). The *B. pertussis* strains containing all of these mutations were derived from our virulent lab strain BP370.

The induction of leukocytosis in mice (4) by the *B. pertussis* strains was measured 4 days after an intravenous (IV) injection of the strains (Fig. 3A). Mice injected with strains producing the native toxin, BP370 and TOX5105, developed a dose-dependent leukocytosis. Curiously, strain TOX5105, which contains an insertion of the *kan^r* gene outside of the toxin structural genes, appeared slightly less potent in promoting leukocytosis. This may reflect a genetic effect of this particular insertion mutation or a physiological effect of the *kan^r* gene product on toxin export or assembly. In contrast,

the codon insertion mutant TOX3201, as well as TOX058 and the nontoxinogenic TOX3311, induced essentially no leukocytosis.

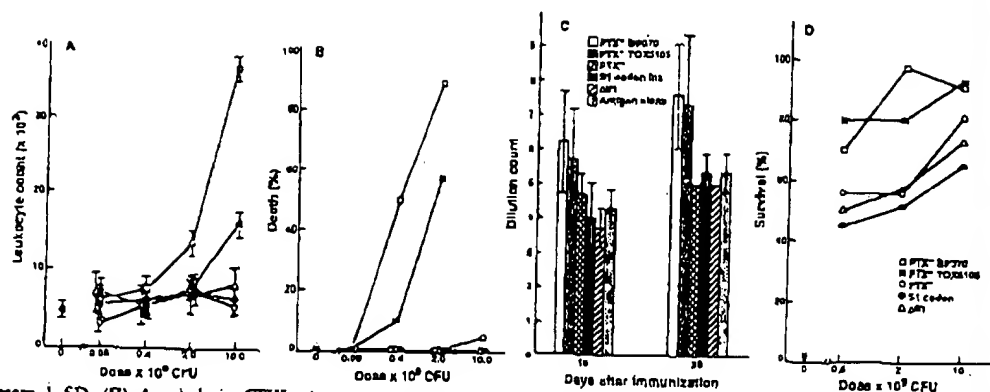
We measured the potentiation of anaphylaxis to two different antigens, chicken egg albumin (EA) in CFW mice (4) and bovine serum albumin (BSA) in BALB/c mice (14, 15). The CFW mice were given concomitant intraperitoneal (IP) injections of EA and heat-killed *B. pertussis*, and sensitization was indicated by a lethal anaphylaxis upon IV challenge with EA 14 days later (Fig. 3B). The native toxin-producing strains BP370 and TOX5105 displayed a dose-dependent sensitizing activity. Similar to the leukocytosis induction, the *kan^r* insertion mutant TOX5105 was less potent. In contrast, the codon insertion mutant, TOX3201, the S1 deletion mutant, TOX058, and the nontoxinogenic TOX3311 were all ineffective in potentiating anaphylaxis. The mice were sensitized to BSA-induced anaphylaxis by injection, for 4 days, on alternating days, with BSA and with the *B. pertussis* strains. Anaphylaxis was induced by injecting mice 5 to 7 days after the sensitization regimen with BSA. In the BSA sensitization challenge, we substituted *B. pertussis* strain TOX5167 for TOX5105. TOX5167 also contains an insertion of the *kan^r* gene, also

Table 1. Potentiation of BSA anaphylaxis and stimulation of histamine sensitivity. BSA anaphylaxis has been referred to as pertussis vaccine encephalopathy (14). *Bordetella pertussis* strains were prepared and administered as reported for histamine challenge (4) and BSA challenge (14). ND, not done.

Strains	CFU × 10 ³	BSA challenge (deaths/total)*	Histamine challenge (deaths/total)*
BP370	10		10/10
	5		ND
	2	17/29 (59%)	10/10
	0.4		10/10
	0.08		3/10
TOX5167	5	26/29 (90%)	ND
TOX5105	9.4		10/10
	2		10/10
	0.4		2/10
	0.08		0/10
	0.01		2/10
TOX3311	9.4		2/10
	5	0/30 (0%)	ND
	2		0/10
	0.4		0/10
	0.08		0/10
TOX3201	10		1/9
	5	0/30 (0%)	ND
	2		0/10
	0.4		0/10
	0.08		0/10
TOX058	9.2		2/9
	5	0/10 (0%)	ND
	2		0/9
	0.4		1/9
	0.08		0/9
PBS only	0.0	ND	0/19
PTX†		71/83 (86%)	ND

*Deaths/total represents the ratio of the number of animals in which the sensitization was lethal to the total number tested. †Pertussis toxin (100 ng, List Biological Laboratories) was administered in place of *B. pertussis* strain.

Fig. 3. Leukocytosis, anaphylaxis, adjuvanticity, and ICC. A key to the strains is presented in (D). The strains were: PTX⁺, BP370; PTX⁺, TOX5105; PTX⁺, TOX3311; S1 codon, TOX3201; and AS1, TOX058. Controls are presented as a *B. pertussis* dose of 0 CFU. (A) Leukocytosis. Leukocytosis was measured (4) in a Coulter counter 4 days after IV injection of *B. pertussis* vaccines (16). Values represent leukocyte count per cubic millimeter and are averages from five animals; bars represent 1 SD. (B) Anaphylaxis. CFW mice were sensitized (4) to EA with an IP dose of 1.0 mg of the antigen and an IV dose of the *B. pertussis* strains. Mice were challenged 14 days later with 1.0 mg of EA given IV. Results are the percentage of mice that died of anaphylaxis. For each graph value the number of animals was ≥ 10 . (C) Adjuvanticity. C57BL/10 SCN mice received 1.0 mg of EA IP and 2×10^9 CFU of heat-killed *B. pertussis* IV on day 0. On day 21, mice received a second IP injection of 5 μ g of EA. Mice were bled on days 16 and 28, and we titrated sera for anti-EA by means of



enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with EA. Mice receiving EA antigen without any *B. pertussis* are indicated as antigen alone. Each value represents three animals; bars represent 1 SD. (D) ICC. Intracerebral challenge protection. Three-week-old CFW mice immunized IP with *B. pertussis* received a second IP injection of 5 μ g of EA. Mice were bled on days 16 and 28, and we titrated sera for anti-EA by means of

outside of the toxin operon (Fig. 1). We found that whereas a dose of 5×10^9 colony-forming units (CFU) of *B. pertussis* strains BP370 and TOX5167 led to a high percentage of sensitization, the *ptx* mutant strains TOX3201, TOX058, and TOX3311, were entirely unable to potentiate an anaphylactic response to BSA (Table 1).

The sensitization of mice to a lethal challenge with the vasoactive amine histamine has also been proposed to reflect a direct action of the B oligomer, in this instance, on the vascular endothelium to increase vascular permeability (8). The sensitizing activities of our *B. pertussis* strains were determined by injecting mice IV with heat-killed *B. pertussis* followed 4 days later by IP challenge with histamine (4). The toxinogenic strains BP370 and TOX5105 increased the sensitivity to histamine in a dose-dependent fashion (Table 1). The mutant strains TOX3201, TOX058, and TOX3311, in contrast, were substantially free of this activity. Thus, our data suggest that with regard to induction of leukocytosis, potentiation of anaphylaxis, and stimulation of histamine sensitivity, a *B. pertussis* strain producing an assembled holotoxin that is reduced in ADP-ribosyltransferase activity is reduced in pathogenic potential to the level of a nontoxinogenic organism.

The adjuvanticity of pertussis toxin in experimental animal models is well documented (4, 28) and may contribute functionally to the efficacy of the whole-cell pertussis vaccine (1, 2). The role of the ADP-ribosyltransferase activity in the adjuvant action, however, has been disputed (8); we therefore tested the mutants for their adjuvant activity in the production of antibodies to the antigen EA (Fig. 3C).

C57BL/10 SCN mice were injected concomitantly with EA and heat-killed *B. pertussis* and were measured 14 days later for anti-EA titers. The toxinogenic parental strain, BP370, exhibited a marked adjuvant action on the production of antibody to EA. The titers were increased further by a small secondary injection of EA given on day 21. The toxinogenic *kan*^r strain, TOX5105, also manifested an adjuvant action, though it was less apparent until after the secondary immunization of EA. In contrast, concomitant injection of EA with the *ptx* mutant strains TOX3201, TOX058, or TOX3311 showed no adjuvant effect after either the primary or secondary injection.

To further investigate the loss of immunostimulation seen with the *ptx* mutants, their ability to protect mice from a lethal intracerebral challenge (ICC) infection with *B. pertussis* (16) was studied. Though it was apparent that the mutations in the S1 subunit gene would interfere functionally with the adjuvant activity of *B. pertussis*, we felt that the assembled and exported CRM3201 holotoxin molecule of TOX3201 might still serve, at least structurally, as an efficacious immunogen. The ICC infection is used to assay the potency of pertussis vaccine preparations in the United States, and involves IP immunization of test mice with whole-cell vaccine preparations, followed 2 weeks later by an ICC with the standard virulent strain of *B. pertussis*, 18323 (16). Both the wild-type and the mutant strains of *B. pertussis* provided a dose-dependent degree of protection against ICC infection (Fig. 3D). However, the dose-response curves for the *ptx* mutant strains TOX3201, TOX058, and TOX3311 were lower than those of the strains producing the native toxin. At the

highest immunizing dose, protection with the native toxin-producing strains approached 100% of a cohort, whereas at similar doses the S1 mutants and the nontoxinogenic mutant induced only about 70% protection. This would seem to indicate that the ADP-ribosyltransferase activity is critical for optimum immunoprotection. An alternative explanation, that the region of the S1 polypeptide that we altered with *ptx*A3201 may be a critical structural epitope, is unlikely since it has been shown that an S1 polypeptide alone containing the native Glu¹⁴⁰-Tyr¹⁴¹-Val¹⁴² region is an inefficient immunogen (29).

Taken together, our results regarding leukocytosis, anaphylaxis, adjuvanticity, and immunoprotection of mice from an ICC infection suggest that the ADP-ribosyltransferase activity of pertussis toxin correlates directly with the immunomodulatory activities of a *B. pertussis* strain. TOX3201 produces an assembled holotoxin with a reduced ADP-ribosyltransferase activity and was reduced in these immunopathologic and immunoprotective activities. These data imply that mutations in the toxin genes that reduce pathogenic activities of a strain such as leukocytosis can also reduce the immunoprotective capacity of the strain. This is an important consideration in the formulation of future pertussis vaccines.

REFERENCES AND NOTES

1. A. C. Wardlaw and R. Parson, *Pharmacol. Ther.* 19, 1 (1983).
2. M. Pirman, *Pediatr. Infect. Dis.* 3, 467 (1984).
3. A. A. Weiss and E. L. Hewlett, *Annu. Rev. Microbiol.* 40, 561 (1986).
4. J. J. Munoz, H. Arai, R. K. Bergman, P. L. Sadowski, *Infect. Immun.* 33, 820 (1981).
5. M. Tamura et al., *Biochemistry* 21, 5516 (1982).

6. G. M. Bokoch, T. Katada, J. K. Northrup, E. L. Hewlett, A. G. Gilman, *J. Biol. Chem.* 258, 2072 (1983); T. Katada, M. Tamura, M. Ui, *Arch. Biochem. Biophys.* 224, 290 (1983); T. Murayama and M. Ui, *J. Biol. Chem.* 258, 3319 (1983).
7. M. Tamura et al., *J. Biol. Chem.* 258, 6756 (1983).
8. M. Ui, K. Nogimori, M. Tamura, in *Protein Tyrosine Kinase*, R. D. Sekura, J. Moss, M. Vaughan, Eds. (Academic Press, Orlando, FL, 1985), pp. 19-43; K. Nogimori et al., *Dev. Biol. Stand.* 61, 51 (1985).
9. M. Gronstrom, G. Gronstrom, P. Gilman, P. Askelof, *J. Infect. Dis.* 151, 646 (1985).
10. Y. Sato, H. Kuriura, H. Fukumi, *Lancet* 1984-I, 122 (1984); G. R. Noble et al., *J. Am. Med. Assoc.* 257, 1351 (1987).
11. J. D. Cherry, *Curr. Clin. Top. Infect. Dis.* 7, 216 (1986).
12. C. Leitch and J. M. Keith, *Science* 232, 1258 (1986); A. Nicolson et al., *Proc. Natl. Acad. Sci. U.S.A.* 83, 4631 (1986).
13. S. I. Morse and J. H. Morse, *J. Exp. Med.* 143, 1483 (1971).
14. L. Steinman et al., *Proc. Natl. Acad. Sci. U.S.A.* 82, 8733 (1985).
15. J. J. Munoz, M. G. Peacock, W. J. Hadlow, *Infect. Immun.* 55, 1004 (1987); K. Redhead, A. Robinson, L. A. E. Ashworth, M. Melville-Smith, *J. Biol. Stand.*, in press.
16. P. Kendrick, G. Eldering, M. K. Dixon, J. Munoz, *Am. J. Public Health* 97, 803 (1947); P. Kendrick, E. L. Updyke, G. Eldering, *ibid.* 39, 179 (1949).
17. F. Hedstrom, M. So, B. J. McCarthy, *Proc. Natl. Acad. Sci. U.S.A.* 76, 6012 (1978).
18. W. J. Black and S. Falkow, *Infect. Immun.* 55, 2465 (1987).
19. S. Stibitz, W. J. Black, S. Falkow, *Gene* 50, 133 (1986).
20. S. F. Carroll et al., *Proc. Natl. Acad. Sci. U.S.A.* 82, 7237 (1985); S. F. Carroll and R. J. Collier, *J. Biol. Chem.* 262, 8707 (1987).
21. P. Y. Chou and G. D. Fasman, *Adv. Enzymol. Relat. Areas Mol. Biol.* 47, 45 (1978).
22. Y. Sato, J. L. Cowell, H. Sato, D. G. Burtyn, C. R. Mandark, *Infect. Immun.* 41, 313 (1983); R. D. Sekura, F. Fish, C. R. Mandark, B. Meade, Y. Zhang, *J. Biol. Chem.* 258, 14647 (1983).
23. U. K. Laemmli, *Nature (London)* 227, 680 (1970).
24. W. N. Burnette, *Anal. Biochem.* 112, 195 (1981).
25. P. A. Watkins et al., *J. Biol. Chem.* 260, 13478 (1985).
26. A. Oka, H. Sugawara, M. Takanami, *J. Mol. Biol.* 147, 217 (1981).
27. W. J. Black et al., in preparation.
28. A. Robinson and L. I. Irons, *Infect. Immun.* 40, 523 (1983).
29. A. Nicolson, A. Bartoloni, M. Perugini, R. Rappuoli, *ibid.* 56, 963 (1987).
30. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
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Immunotherapy of the Nonobese Diabetic Mouse: Treatment with an Antibody to T-Helper Lymphocytes

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Spontaneous diabetes mellitus was blocked in nonobese diabetic mice by treatment with a monoclonal antibody against the L3T4 determinant present on the surface of T-helper lymphocytes. Sustained treatment with the monoclonal antibody led to cessation of the lymphocytic infiltration associated with the destruction of the insulin-producing β cells. Moreover, the mice remained normoglycemic after the antibody therapy was stopped. These studies indicate that immunotherapy with monoclonal antibodies to the lymphocyte subset may not only halt the progression of diabetes, but may lead to long-term reversal of the disease after therapy has ended.

THERE IS INCREASING EVIDENCE that human insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease and that IDDM results from immune destruction of the insulin-producing β cells normally found in the islets of Langerhans (1). Nonobese diabetic (NOD) mice spontaneously develop diabetes (2) resembling human IDDM. As in human IDDM, the NOD mice have progressive lymphocytic infiltration into the islets (insulinitis) before the expression of overt diabetes (2-4), and cytoplasmic antibodies to islet cells appear in their serum during the development of insulinitis (4, 5). Susceptibility to diabetes in both humans and NOD mice is strongly associated with genes of the major histocompatibility complex (MHC) (6). Overt diabetes is characterized by polyuria, polydipsia, hyperglycemia, and glycosuria, and NOD mice develop acute ketoacidosis, which is fatal unless the mice are treated with insulin (2, 7).

The specific immunologic pathways and

cell types responsible for islet cell destruction in NOD mice are not clearly delineated (8). However, recent studies suggest that the T lymphocyte subset that expresses the L3T4 surface marker is important in the pathogenesis of the disease (9). T lymphocytes of the L3T4 phenotype are a distinct subpopulation of mature T cells that function as helper-inducer cells in the activation of both humoral and cellular immunity (10). The L3T4 lymphocyte subset is responsible for MHC class II-restricted antigen recognition on antigen-presenting cells (11); the human homolog to the murine L3T4⁺ T cell is the CD4⁺ T cell (11). We have been able to block the progression and subsequent expression of overt diabetes in NOD mice by a course of treatment with a monoclonal antibody to L3T4. Such an approach may be feasible for treatment of patients with subclinical manifestations of IDDM, since we show that antibody therapy initiated late in disease progression was effective in reversing the advanced phases of islet cell destruction. Moreover, upon cessation of therapy the mice have remained disease-free without further treatment.

The monoclonal antibody used in these studies, GK1.5, is a cell-depleting antibody. When administered to mice at doses greater than 300 μ g, this antibody causes sustained reduction of more than 90% of the circulating L3T4⁺ cells (12). GK1.5 has been successfully used in vivo as an immunotherapeutic agent to treat other experimental and spontaneous autoimmune diseases, including systemic lupus erythematosus (13), experimental allergic encephalomyelitis (14), and type II collagen-induced arthritis (15). In addition, a single course of this antibody has been shown to allow indefinite acceptance of transplanted allogeneic murine islets of Langerhans (16). GK1.5 and other antibodies to L3T4 are particularly suitable for serotherapy, since these reagents can suppress the humoral immune response (12, 17) and induce tolerance to select protein antigens, including the monoclonal antibody to L3T4 itself (17).

When NOD mice are 30 to 50 days old, mononuclear cells begin to infiltrate the perivascular and periductal areas around the

Table 1. Prevention of diabetes in NOD mice by long-term treatment with GK1.5. Rat monoclonal antibody GK1.5 (immunoglobulin G2b) to mouse L3T4, purified from ascites fluid, was administered intraperitoneally to 90- to 110-day-old NOD female mice. Incidence of diabetes is shown as the ratio of the number of diabetic mice to total number of mice in the group at 260 days of age.

Amount of GK1.5 administered (μ g)	Incidence of diabetes	Time of onset of diabetes (days)
600	18/21	157 \pm 83
600, then 100 weekly	2/25	156 \pm 43
None	29/35	173 \pm 42

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